β -ADRENERGIC STIMULATION INCREASES PROTEIN PHOSPHORYLATION AND SKELETAL MUSCLE α–ACTIN mRNA ABUNDANCE IN C_2C_{12} MYOTUBES

By

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ABSTRACT

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β-adrenergic agonist (βAA) stimulation of skeletal muscle increases expression of muscle specific genes and muscle hypertrophy. The exact mechanism through which these actions occur remains unresolved. The objective of this study was to investigate the mechanism of βAA-stimulated muscle hypertrophy using the immortalized mouse hind limb skeletal muscle cell line, C2C12. The myogenic phenotype of C2C12 cells was characterized through assay of skeletal muscle specific gene expression (skeletal α-actin, SKA), protein (sarcomeric myosin, SM), and enzyme activity (muscle creatine kinase, MCK). Detection of SKA messenger ribonucleic acid (mRNA) abundance in C2C12 myotubes was accomplished using a SKA isoform specific, sequence verified, 105 bp digoxygenin labeled, polymerase chain reaction (PCR) generated probe homologous to 3591 bp-3696 bp of the 3' untranslated region (UTR) region of the mouse SKA gene.

Abundance of SKA mRNA was expressed per unit of 18S ribosomal ribonucleic acid (rRNA). The appearance of SKA mRNA occurred approximately 48 hours post confluence, increased as cells differentiated and fused to form myotubes, and paralleled the appearance of SM detected using an anti-SM antibody. A dose-response study suggested that, for the stimulation of SKA, the optimal concentration of the synthetic βAA, isoproterenol (ISO), was approximately 10^{-5} M. Further experiments revealed that the response to stimulation is maximal at 72 hours post stimulation and is depressed by the addition of the non-selective beta adrenergic receptor (βAR)-antagonist, propranolol (PRO), or protein kinase A (PKA) inhibitor, HA1004. The effects of βAA stimulation of PKA mediated phosphorylation (~P) of key proteins were determined using an anti-P-threonine antibody. Immunoblots of cell extracts revealed an ~90 kilo-Dalton (kDa) βAA-responsive, developmentally regulated, cytoplasmic protein in cell extracts of C2C12 myotubes. The identity of the protein remains undetermined.

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LIST OF ABBREVIATIONS

Ab	antibody
AC	adenylyl cyclase
anti-dig-Fab	anti-digoxygenin Fab
AP	alkaline phosphatase
AP-1	activator protein-1
Asp	aspartic acid
ATCC	Americain tissue culture collection
βAA	beta-adrenergic agonist
βAR	beta-adrenergic receptor
βARK	beta-adrenergic receptor kinase
BLAST	basic logical alignment search tool
bp	base pair
BSA	bovine serum albumen
CAA	cardiac α-actin
cAMP	cyclic adenosine-monophosphate
CLEN	clenbuterol
CRE	cAMP response element
CREB	cAMP response element binding protein
CTOX	cholera toxin
DAG	diacyl glycerol
DEPC	diethyl pyrocarbonate
DMEM	Dulbeccos modified eagles medium
DNA	deoxyribonucleic acid
EPI	epinephrine
F-actin	filamentous actin
FBS	fetal bovine serum
FOR	forskolin
G-actin	globular actin
GDP	guanine diphosphate
G_i	G-protein (inhibitory)
Glu	glutamic acid
G_s	G-protein (stimulatory)
GTP	guanine triphosphate
HA1004	N-(2-guanidinoethyl)-5-isoquinolinesulfonamide

HRP	horseradish peroxidase
H7	1-(5-isoquinolinylsulfonyl)-2-methylpiperazine
IP_3	inositol triphosphate
ISO	isoproterenol
JSR	junctional sarcoplasmic reticulum
kb	kilobase
kDa	kilo-Dalton
Leu	leucine
MAPK	mitogen-activated protein kinase
MCK	muscle creatine kinase
Met	methionine
MCE	mercaptoethanol
mRNA	messenger ribonucleic acid
NFEP	anti-phosphatase buffer
NOREPI	norepinephrine
~P	phosphorylate/d/ion
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDE	phosphodiesterase
PDE-I	4(3,butoxy,4-methoxybenzyl)imidizolidin-2-one
PE	phorbol ester
pI	isoelectric point
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	cAMP dependent protein kinase
PKC	protein kinase C
PRO	propranolol
RAC	ractopamine
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
Ser	serine
SKA	skeletal α-actin
SM	sarcomeric myosin
SR	sarcoplasmic reticulum
SSC	sodium chloride/sodium citrate buffer
ST	somatotropin
TBE	tris/EDTA buffer
Thr	threonine
TPA	tissue plasminogen activator
2-D	two-dimensional
UTR	untranslated region
UV	ultraviolet
X-Gal	5-bromo-4-chloro-3-indoyl-β-galactosidase

INTRODUCTION

Consumer demand for leaner food products and a recognition of this market by producers have fostered continued research on factors affecting lean and fat deposition in food-producing animals. Growth rate and body composition of livestock can be optimized to meet consumer needs for a leaner food product and to improve the efficiency of meat-animal production (Wray-Cahen et al., 1998). The field of study in animal science now referred to as growth biology has been concerned with the mechanisms of protein and fat deposition in food-producing animals (Bergen et al., 1996). Increasing the productivity of livestock species can be interpreted as increasing lean gain while depressing fat gain, or faster more efficient gain. Depending on the species, 50-70% of production costs are associated with feed. As a result, much research has been aimed at maximizing feed-conversion efficiency (Wray-Cahen et al., 1998). The feed-conversion ratio represents the interplay between the rate of body gain, the nature of the tissue deposited, and the inefficiencies of digestive processes (Wray-Cahen et al., 1998). Approximately 60% of total carcass weight is represented by skeletal muscle mass (Mulvaney, 1981). Skeletal muscle represents the primary economic product in a livestock production system; therefore, increasing productivity may increase economic gains. My long term goal is to investigate the mechanism by which growth-promoting hormones and other growth-promoting substances affect skeletal muscle protein accretion. This should lead to

strategies that improve the efficiency of lean tissue growth and would improve the economics of food animal production.

Identification and interpretation of the responses to a variety of strategies utilized to modify body composition have been extensive. Regardless of the strategy applied, all attempt to shift the priority of nutrient allocation toward lean tissue deposition while simultaneously decreasing adipose tissue accretion (Hammond, 1952, Wray-Cahen et al., 1998). Animal scientists have used a variety of strategies to increase animal productivity, from selective breeding to the use of exogenous agents. By the middle of the 1980's, availability of species-specific recombinantly produced somatotropin (ST), as well as orally active β-adrenergic agonists (βAA), encouraged a phenomenal amount of research on administration of ST and feeding βAA to animals (Bergen and Merkel, 1991). Extensive research has been conducted on the physiological effects and biochemical mode of action of repartitioning agents on protein and fat metabolism in meat-producing animals. Partitioning agents, such as ST or βAA, have increased rate of gain, improved efficiency of feed utilization, depressed fat deposition, and enhanced lean gain in livestock species (Asato et al., 1984, Baker and Kiernan, 1983, Bergen and Merkel, 1991, Muir, 1988) (Table 1). Administration of exogenous agents to animals may improve the efficiency of lean tissue gain. A better understanding of the repartitioning mechanism is essential to (1) utilize nutrient repartitioning strategies to their potential and (2) address more specific questions regarding mechanism of action. Maximal lean gain translates into decreased feed/gain, decreased waste, decreased time to market, and finally a greater

Table 1 β-adrenergic activity in livestock

Characteristic	Poultry	Ruminant *	Swine
Carcass protein (% increase)	6	10	4-8
Loineye area (% increase)	-	15-20	9-15
Carcass fat (% decrease)	4-8	20-30	10-16
Back fat (% decrease)	-	20-50	10-17
Abdominal fat (% decrease)	2-8	20-45	-

* Sheep and cattle data

The table represents the effects of β-adrenergic activity in altering body composition across livestock species. The β-adrenergic agonists dramatically alter body composition toward that of a leaner animal (Muir, 1988).

return to the producer. Presently, the molecular mechanism by which these exogenous agents are acting is not completely understood.

The focus of my dissertation research is to investigate the mechanism by which βAA affect skeletal muscle growth at the pretranslational level, as well as through signal transduction pathway mediated phosphorylation.

SKA represents a myofibrillar protein and a unique candidate for studying skeletal muscle growth. Despite the high degree of homology across the nucleic acid and amino acid sequences of the six actin isoforms, the 3' UTR of these genes may confer specificity. The specificity in the 3' UTR may allow for study of individual isoforms. Unlike actin probes used previously, the isoform-specific probe will allow for a more precise measurement of a specific actin isoform.

The initial objective was to isolate and characterize a SKA PCR generated probe homologous to a region of the 3' UTR of the mouse SKA gene. The SKA specific probe will be useful for experimental analysis of the nuclear events preceding translation of SKA in C2C12 myotubes.

Feeding of βAA to livestock species has been reported to increase muscle mass, total muscle protein, total RNA, fractional synthesis rates and the expression of myofibrillar protein genes (Bergen et al., 1989, Helferich et al., 1990, Smith et al., 1989, Koohmaraie et al., 1991). Helferich et al., (1990) report that feeding of the βAA, ractopamine (RAC),

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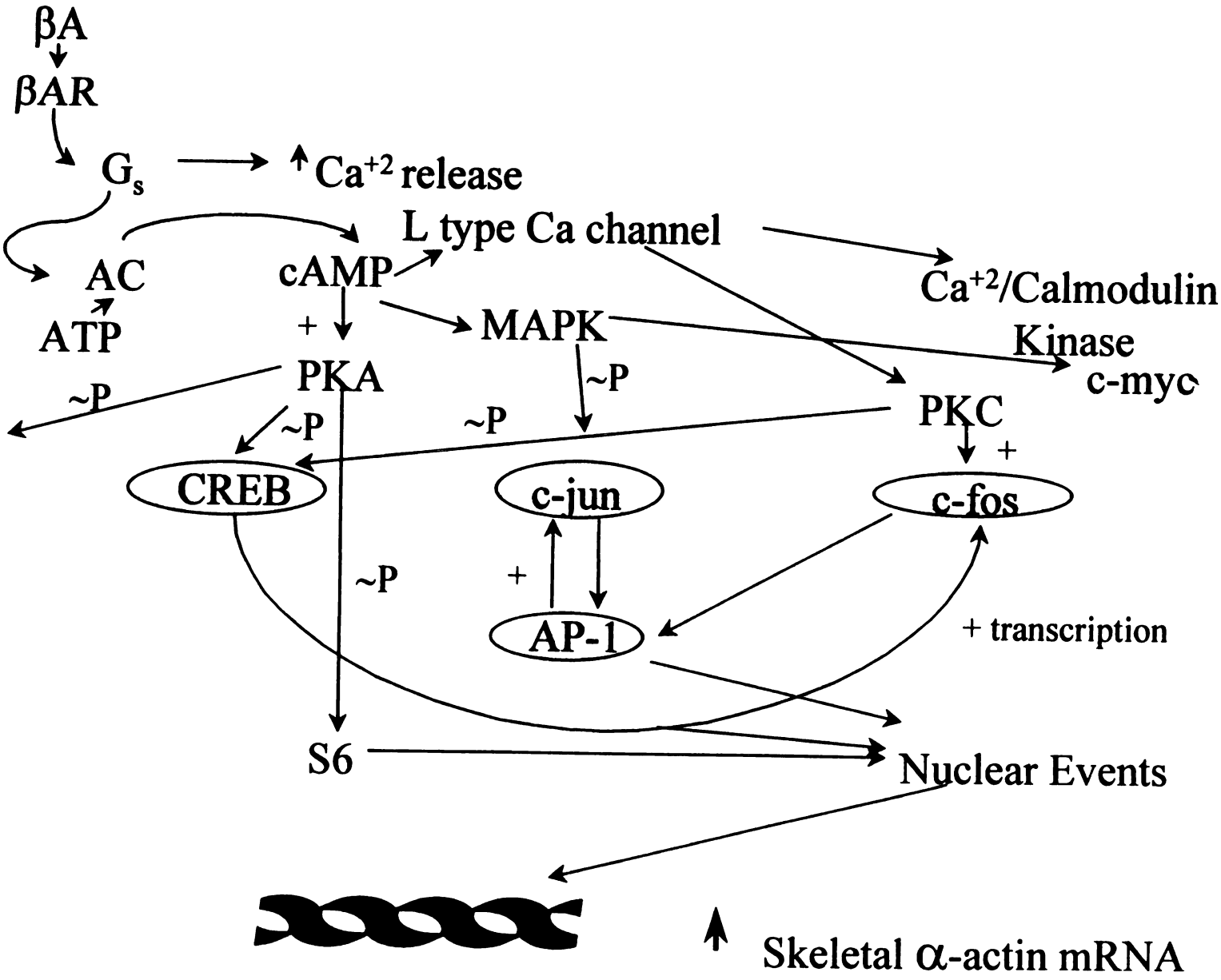
had increased SKA mRNA abundance in the longissimus dorsi muscle of finishing pigs, approximately two-fold above control animals.

The second objective was to investigate the effect of βA stimulation of C2C12 myotubes on SKA mRNA abundance using a SKA isoform-specific probe. Furthermore, although the effects of feeding the βAA, RAC, to finishing pigs were clear, the in vivo study did not allow for an examination or verification of the signal transduction pathway/s involved in increased SKA mRNA abundance. The sub-objective of these sets of experiments was to examine and verify the mechanism of βAA stimulated increased SKA mRNA abundance in C2C12 myotubes.

Extracellular mediated events, such as βAA stimulation, may act through a complex interplay across signal transduction cascades. A major regulatory point in most signal transduction cascades is a specific kinase activated in response to stimulation at a particular membrane-bound receptor. Many kinases, across cascades, may converge on the same transcriptional regulator and may act in concert in activation of responsive genes (Figure 1). Many of these transcriptional regulators (trans-factors) are phosphoproteins. The phosphoproteins are ~P by the activated kinase in the appropriate cascade. Particular segments of DNA in the promoter region of the SKA gene suggest that several trans-factors (c-fos, c-jun, AP-1, CREB) may bind the DNA and may initiate transcription. Several of these known trans-factors have been reported to be responsive to stimulation by multiple signal transduction cascades.

Figure 1 Composite of signal transduction cascade interactions and skeletal α-actin mRNA abundance

The diagram presented in figure represents a composite of signal transduction pathway cascades and their proposed interaction, termed "cross-talk". The pathways presented may converge on several transcriptional regulatory proteins (CREB, c-fos, c-jun) which have been implicated in activity at the skeletal α-actin promoter. Several transcriptional regulatory proteins (trans-factors) may be required in a complex interaction to initiate gene activation.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Skeletal α-actin mRNA

The final objective was to examine cell extracts of C2C12 myotubes to (1) identify a ~P response to βAA stimulation and (2) identify those responsive phosphoproteins. It is anticipated that the responsive proteins may be phosphoproteins involved in the βAA-stimulated muscle hypertrophic response in skeletal muscle.

Investigation of βAA-stimulated SKA mRNA abundance and ~P response in C2C12 myotubes may be critical in defining the mechanism and introducing new potentials related to βAA manipulation of animal growth.

LITERATURE REVIEW

Myogenesis

Myogenesis is the formation of muscle fibers from mononucleated cells (Grant and Helferich, 1991). The development of skeletal muscle is a precisely orchestrated event occurring primarily by hyperplasia, prenatally. The number of muscle cells increases after birth. Postnatal growth occurs primarily through hypertrophy of existing fibers.

The development of skeletal muscle begins soon after conception and can be divided into four stages (1) determination, (2) commitment, (3) differentiation and (4) innervation.The first stage is characterized by the irreversible transformation of the multipotential mesodermal precursor cells to the myogenic lineage, termed determination. Determination of precursor cells to myoblasts restricts the myoblasts to express muscle-specific genes upon differentiation (Grant and Helferich, 1991).

Secondly, the committed cells proliferate until differentiation signals become present. Commitment to the myogenic lineage is accomplished through the coordinate expression of various myogenic regulatory factors. The myogenic regulatory factors act as transcription factors capable of initiating expression of various genes which are essential for differentiation to the myogenic phenotype. Konieczny and Emerson (1984) provided

evidence for a family of regulatory genes that control the determination of precursor cells to muscle cell lineages. The third stage is differentiation.

At differentiation, presumptive myoblasts withdraw from the cell cycle and begin to express the muscle-specific proteins and fusion to myotubes occurs. During differentiation and fusion of mononucleated myoblasts to multinucleated myotubes, environmental stimuli can alter the phenotypes that are expressed, resulting in fibers that are fast, slow, or mixed (fast/slow)(Grant and Helferich, 1991). Different isoforms of contractile proteins are synthesized as differentiation progresses and, in relation to myosin, may be related to fiber type (Grant and Helferich, 1991, Robbins et al., 1986).

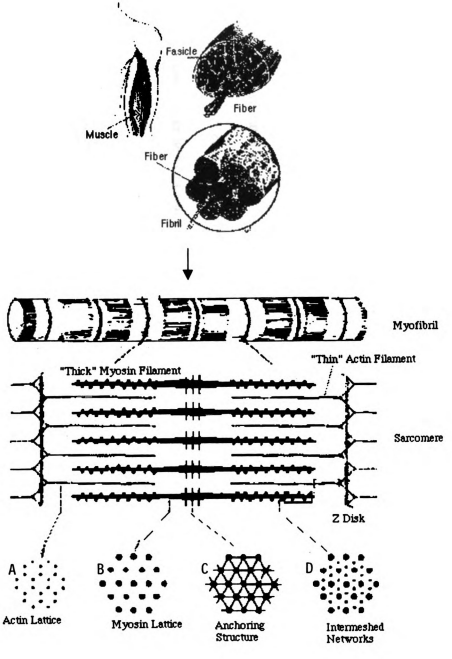
The last stage is recognized by innervation which is essential for proper functioning of the muscle as a tissue and growth.

Skeletal Muscle Structure

The highly ordered structure of skeletal muscle from the gross structure to the molecular level is depicted in Figure 2. The basic unit of the contractile process in striated muscle is the sarcomere (Squire, 1981, Richter et al., 1989) which is composed of thick and thin filaments tandemly arranged in the myofibril. Myofibrillar proteins, which represent more than 50% of the total protein content of skeletal muscle, include the structural proteins of the thick and thin filaments, as well as those that regulate contraction (Grant and Helferich, 1991). The sarcomere is composed of 10-15 myofibrillar proteins. Of the

Figure 2 Skeletal muscle structure

The figure represents the complex organization of skeletal muscle structure from the gross macromolecular level to the intricate molecular level. Organization at the level of the myofibril is presented to illustrate the positioning of actin and myosin in a single contractile unit, the sarcomere. Depicted in A, B, C, and D are representative cross-sections of the contractile unit at longitudinal locations along the sarcomere. [Online image] Available http://ortho84-13.ucsd.edu/MusIntro/Fibril.html, September 10, 1998.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myofibrillar proteins, only myosin, actin, troponin, and tropomyosin participate directly in the contractile event (Richter et al., 1989). Myosin is the predominant myofibrillar protein and accounts for 43% of the total myofibrillar protein. SKA, the second most abundant myofibrillar protein represents 22% of the total myofibrillar protein (Yates and Greaser, 1983, Grant and Helferich, 1991).

Myofibrillar Gene Expression

Regulation of myofibrillar gene expression seems to occur at the transcriptional level, with some of their RNA products exhibiting alternative exon splicing in the generation of multiple protein isoforms (Richter et al., 1989). Some of these primary RNA transcripts are generated by differential initiation at alternate promoters (Nabeshima et al., 1984, Periasamy et al., 1984, Robert et al,. 1984, Richter et al., 1989). Muscle protein diversity can further be manifested by termination of primary transcripts at alternative 3' untranslated sequences (Basi et al., 1984, Ruiz-Opazo et al., 1985, Bernstein et al., 1986, Rozek and Davidson, 1986, Richter et al., 1989). Two possible molecular mechanisms may explain the diversity in protein isoforms: (1) the selective expression of one of the genes from a multigene family, dependent upon tissue and developmental stage-specific factors (Richter et al., 1989) , and (2) the generation of different protein isoforms from a single gene (Nadal-Ginard et al., 1987, Richter et al., 1989). Both mechanisms involve specific cis- or trans- acting factors (Richter et al., 1989). A cis-acting factor refers to a DNA locus that affects the activity of DNA sequences on its own molecule of DNA. A trans-acting factor refers to a diffusable product able to act on all receptive sites in the

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cell (Richter et al., 1989). The components of the skeletal muscle contractile apparatus are products of gene families that each encode structurally and functionally related proteins (Cox et al., 1990) and include myosin heavy chain, myosin light chain, actin, tropomyosin, and troponin (Nadal-Ginard et al., 1982). The differential expression of these genes follows a complex program during muscle development (Cox et al., 1990). Myoblasts from skeletal muscle can be cultured in vitro, either as primary cultures or muscle cell lines, and will fuse spontaneously to form myotubes (Cox et al., 1990, Buckingham, 1985). The fusion of proliferating myoblasts to form myofibers is accompanied by the developmental regulation of genes encoding a structurally diverse group of proteins that form the muscle sarcomere (Muscat et al., 1987). The change in contractile protein phenotype which accompanies the myoblast-to-myotube transition and subsequent modulation of transcript levels may be achieved by regulating rates of transcription and/or by changes in export and stability of the RNA's themselves (Cox et al., 1990). The morphological differentiation is accompanied by the accumulation of sarcomeric contractile protein mRNA's, i.e. SKA, and the synthesis of corresponding protein, while non-muscle actin isoforms, β and γ, are down-regulated (Alwine et al., 1979, Devlin et al., 1978, Gunning et al., 1987, Shani et al., 1981).

Actin

Actin is a globular-shaped molecule, approximately 5.5 nm in diameter, composed of a single polypeptide chain of 374 amino acids with a molecular weight of 42 kDa (Elizinga et al., 1973, Skjaerlund, 1993). The spherical molecule is termed G-actin (for globular

actin) and as such constitutes the monomeric (single molecule) form of actin. The fibrous nature of the actin filament occurs because the G-actin monomers link to form F-actin (fibrous actin) as shown in Figure 1. In F-actin, the G monomers are linked together in strands, much like beads on a string of pearls. Two strands of F-actin are spirally coiled around one another to form the "super-helix" that is characteristic of the actin filament. The actin filament in association with two other myofibrillar proteins (tropomyosin and troponin), form the thin filament involved in the contractile apparatus.

Actins are highly conserved, ubiquitous proteins found in eukaryotes involved in cell motility and in the maintenance of cell structure (Vandekerkhove et al., 1986, Mayer et al., 1984, Garner et al., 1989, Pollard and Weihing, 1974). In mammals, the actins are encoded by a multigene family giving rise to at least six different isoforms, each the product of a single gene, expressed in a tissue-specific manner or in the case of the cytoskeletal actins, ubiquitously (Barton et al., 1987, Vandekerkhove et al., 1986, Garner et al., 1989, Minty et al., 1981).

The actin isoforms can be separated by isoelectric focusing. Separation of α, β, and γ isoactins on a two-dimensional (2-D) gel is possible due to an alkaline stretch of amino acids in the β and γ isoforms (Obinata et al., 1981). Skeletal and cardiac isoforms have very similar isoelectric points which is explained by their highly conserved amino acid sequence (Vandekerckhove and Weber, 1987). Amino acid sequence analysis confirmed that the actins are greater than 90% homologous in amino acid sequence (Minty et al.,

1981). Few differences have been reported in amino acid sequence for the same actin isoform across species (Vandekerckhove et al., 1986). The six different isoactins can be classified according to the tissues in which they appear as the predominant isoforms (Vandekerckhove et al., 1986). Two isoforms are present in the cytoplasmic microfilaments of most or all cell types, while other actin isoforms are found in the contractile apparatus of skeletal, cardiac, and, smooth muscle (Mayer et al. 1984, Minty et al., 1981). Non-muscle cells express two homologous isoactins generally referred to as β and γ-cyotplasmic actins (Vandekerckhove et al., 1986). Smooth muscle tissues express two closely related isoforms referred to as γ smooth muscle and α smooth muscle actin (Vandekerkhove et al., 1986). The γ type is the major form in visceral tissue, while the α type appears as the major form in vascular smooth muscle (Vandekerkhove et al., 1986). In smooth muscle, the γ and α types differ in about 20-23 residues from the non-muscle variants and are similar to, but distinct from, the major isoforms expressed in striated muscle (Vandekerkhove et al., 1986). Cardiac and skeletal striated muscles contain characteristic actin isoforms which have identical molecular weights and isoelectric points, and only the complete amino acid sequence has revealed differences between the major isoform expressed in fast and slow twitch skeletal muscle and that in the rat heart ventricle (Vandekerkhove et al., 1986). No fiber-type-specific or developmental isoforms of skeletal α-actin have been found. The skeletal muscle type α-actin, differs from the cardiac variant by a Glu to Asp exchange at residues 2 and 3 and by exchanges of Met to Leu and Ser to Thr at positions 299 and 358, respectively. The

two latter exchanges are also typical of smooth and non-muscle actins (Vandekerkhove et al., 1986).

Pairs of these genes are differentially expressed throughout development and in adult tissues (Garner et al., 1989). Two sarcomeric actins, cardiac α-actin (CAA) and SKA have been defined (Garner et al., 1989). They are coexpressed at high levels during the development of striated muscle, but in the adult, the CAA and SKA isoforms predominate in cardiac and skeletal muscles, respectively (Garner et al., 1989). SKA and CAA isoforms migrate at 1.6 kb on an agarose gel while the non-muscle actin isoforms, β and γ, migrate at approximately 2.1 kb on an agarose gel (Garfinklel et al., 1982, Mayer et al., 1984, Minty et al., 1981).

Actin Isoform Switching

Regulation of α-actin expression occurs at the transcriptional level in a tissue specific manner (Vandekerckove et al., 1979, Richter et al., 1989). The actin genes are not linked in mammalian genomes (Czosnek et al., 1983, Gunning et al., 1984, Richter et al., 1989) and are unlinked to any other sarcomeric protein genes (Czosnek et al., 1983). Contrary to the non-muscle actin isoforms, CAA and SKA are synthesized in large amounts but with distinct tissue-specific and developmental patterns of expression (Bains et al., 1984, Devlin et al., 1979, Mohun et al., 1986, Nudel et al., 1986, Muscat et al., 1987, Minty et al., 1982, Gunning et al., 1983, Singer et al., 1978). In replicating myogenic cells, β-actin is the predominant cytoplasmic isoform (Richter et al, 1989). After fusion initiates,

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synthesis of β-actin decreases and the sarcomeric α-actins begin to appear (Seiler-Tuyns et al., 1984). Older myotube cultures may undergo some of the changes in actin and myosin gene expression seen in vivo, and this situation can be manipulated to some extent in myogenic C_2 cell line (Pinset et al., 1988, Weydert et al., 1987).

Cox et al.(1990) reported that there is a sequential transition in transcription and RNA accumulation during myotube maturation which reflects the pattern of expression found during development in vivo from that of predominantly CAA to predominantly SKA. It has been suggested that one or the other isoform predominates in adult tissue to give rise to a selective functional advantage for that particular tissue. A precise fine tuning of gene expression may not be necessary or desirable at crucial stages of development when it is more important to generate large quantities of muscle proteins (Richter et al., 1989, Buckingham et al., 1985). During development, CAA appears to be the dominant isoform in both skeletal muscle and in heart (Minty et al., 1982). In adult human skeletal muscles, the SKA isoform predominates, but the CAA mRNA still represents 5% of the sarcomeric actin transcripts (Gunning et al., 1983). Along the same lines, in adult heart muscle, the CAA isoform predominates (Mayer et al., 1984, Waslyk et al., 1980). In primary chicken and human myoblasts differentiating in culture, similar patterns of α-actin isoform mRNA accumulation are observed (Hayward et al., 1982, Gunning et al., 1987). CAA predominates, but SKA mRNA is transiently present at moderate levels and, subsequently, at 10 to 15% of adult muscle levels. In the frequently studied, immortalized, rodent myogenic cell lines, C_2C_{12}, L_8, and L_6, the patterns of sarcomeric α-

actin mRNA differ substantially and are different from the patterns seen in primary cells (Muscat et al., 1987). CAA transcripts accumulate to extraordinary levels in C_2C_{12} cells, but, are not detectable in L_6 cells and only accumulate to 10 to 15% of adult levels in L_8 cells. SKA transcripts in these cell types have similar patterns of accumulation and slowly rise to 10 to 15% of adult levels (Muscat et al., 1987, Bains et al., 1984, Hayward et al., 1982, Hickey et al., 1986, Minty et al., 1986). Functionally, the contractile apparatus in skeletal muscle and cardiac muscle appears to be the same regardless of the fact that different actin isoforms predominate.

Skeletal α-actin

Skeletal α-actin (SKA) represents a highly conserved muscle-specific protein in skeletal muscle. The fact that SKA is developmentally regulated makes SKA a unique candidate for studying muscle growth. Actin is the second most abundant contractile protein in skeletal muscle myofibrils and represents 22% of total myofibrillar protein (Skjaerlund et al., 1993). Even though myosin is more abundant, there have been over 12 myosin isoforms identified in skeletal muscle which presents a greater complication than just studying one actin isoform (Skjaerlund, 1993). The skeletal muscle isoform, SKA, represents greater than 95% of all actin present in mature skeletal muscle (Skjaerlund et al., 1993). The inherent problem with using SKA mRNA abundance as an index of myofibrillar gene expression lies in its highly conserved amino acid and nucleic acid sequence. The nucleic acid sequence across actins is less than 2% divergent while the amino acid sequence has been shown to be less than 20% divergent, a sequence diversity

due to the degeneracy of the genetic code (Buckingham et al., 1984, Buckingham, 1985. Skjaerlund, 1993). Nucleic acid probes for actin tend to cross-react with other actin isoforms, making data difficult to interpret. Nucleic acid homology comparisons across actin isoforms indicate that the 3' UTR may confer specificity across isoforms (Mayer et al., 1984, Minty et al., 1981, Wettenhall et al., 1982).

Transcriptional Regulation of Skeletal α-actin

Two upstream cis-acting elements that modulate the tissue specific transcription of the human CAA gene may represent similar sequences within the SKA promoter (Minty et al., 1986, Minty et al., 1986, Richter et al., 1989). These regions may be modulated by tissue-specific transcription factors (trans- factors) in skeletal muscle as they are in cardiac muscle. Muscat and Kedes (1987) revealed the complexity of the SKA promoter reporting specific domains which are responsible for gene transcription. The proximal domain (positions -153 to -87) is critical for tissue-specific expression and regulation in two myogenic cell systems (Muscat and Kedes, 1987). The cis-acting sequences 3' of position -87 interact with factors present in both myogenic and fibroblastic cells and appear to define or act as the major component of the basal promoter domain. The distal 5' domain from positions -1300 to -626 and the proximal domain from positions -153 to -87 respond to muscle-specific factors and possibly modulate transcription in a synergistic fashion in C_2C_{12} cells but not in L_8 cells.

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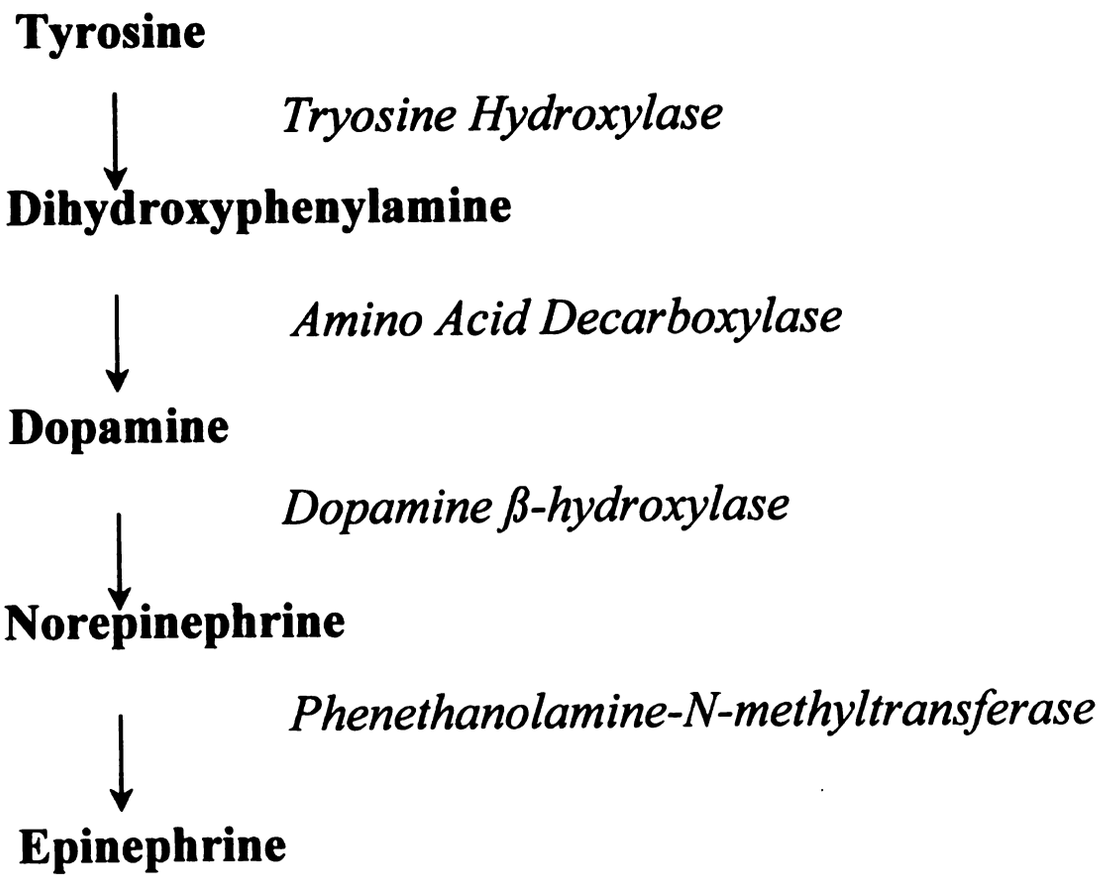
The central domain between positions -626 and -153, although not required in either cell line, has a positive role in augmenting expression in L8 cells (two- to threefold) but not in C_2C_{12} cells. Muscat and Kedes (1987) reported that these elements appear to be differentially utilized for maximal expression in different myogenic cells. Subsequently, the number and functional diversity of the regulatory domains and the distance they span raise the issue of their roles in the distinctive patterns of SKA mRNA accumulation during myogenesis in rodent cell lines and primary cell lines (Bains et al., 1984, Hickey et al., 1986, Minty et al., 1986, Hayward et al., 1982, Gunning et al., 1987). Their observations are compatible with the possibility that the particular combination of domains involved in vivo depends on the availability or relative levels of trans-acting factors in each cell type and the accessibility of the different domains to these factors. It is possible that the particular cell lines may differ in the kind and amount of trans-acting factors produced, emphasizing the complexity of the promoter region and the variety of interactions which regulate its function.

β- Adrenergic Agonists

The physiological βAA are represented by the catecholamines, epinephrine and norepinephrine. They are sequentially synthesized from the amino acid tyorsine in a series of hydroxylation, decarboxylation, and methylation reactions as (3,4)-dihydroxy derivatives of phenylethylamine (Figure 3) (Axelrod, 1971, Ungar et al., 1983). Synthetic βAA compounds exist which exhibit similar pharmacological and chemical properties to those of the endogenous catecholamines (Figure 4). These compounds can act through

Figure 3 Catecholamine biosynthesis

The naturally occurring catecholamines epinephrine and norepinephrine are synthesized in the adrenal medulla from the amino acid tyrosine in a series of hydroxylation, decarboxylation, and methylation reactions.


Tyrosine
Tryosine Hydroxylase
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Amino Acid Decarboxylase
Dopamine
Dopamine ß-hydroxylase
Norepinephrine
Phenethanolamine-N-methyltransferase
Epinephrine

Figure 4 Common β-adrenergic agonists

Synthetic β-adrenergic agonists share the common structure of the endogenous catecholamines and may share similar pharmacological properties. The structures of several β-adrenergic agonists are presented to illustrate the similarity in regard to the catechol nucleus and amine side chain between physiological and synthetic anlaogues. β-adrenergic agonists represented include the physiolgical catecholamine, epinephrine and several synthetic β-adrenergic agonists including isoproterenol.

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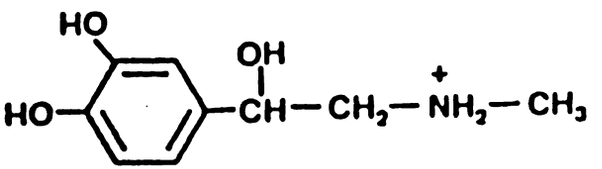

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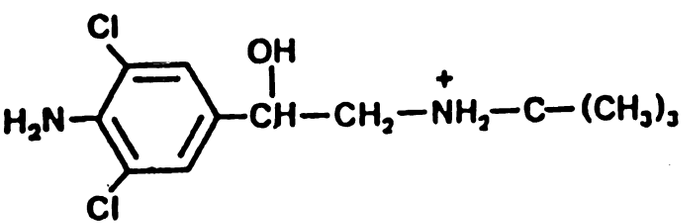

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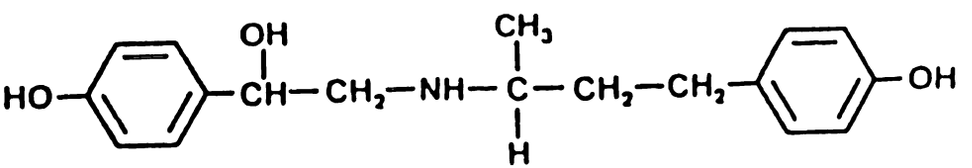
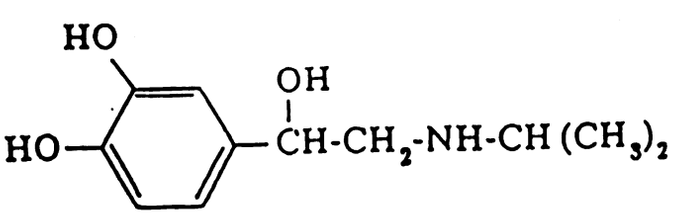


Isoproterenol



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two general classes of receptors termed either α or β, and are classified on receptor binding affinity (Figure 5).

β-Adrenergic Receptors

The βAR is represented in Figure 6 (Hausdorff et al., 1990, Strosberg, 1990,1993, Mills and Mersmann, 1995). The βAR's mediate their responses through a G_s protein. The βAR's have seven membrane-spanning domains and intervening stretches forming intra- and extracellular loops (Strader et al., 1989, Mills and Mersmann, 1995). The transmembrane sequences are more highly conserved between the βAR subtypes than are the intra/extracellular loops (Mills and Mersmann, 1995). The transmembrane helices are arranged cylindrically to form a binding pocket for the agonists and antagonists (Strader et al., 1989, Blin et al., 1993, Mills and Mersmann, 1995). Agonists and antagonists bind in a similar orientation in the binding pocket. Activation of the G_s is preceded by a conformational change in the receptor due to association of key amino acids of the ligand interacting with the receptor suggesting that only subtle differences separate a functional agonist from an antagonist (Mills and Mersmann, 1995). The idea of a partial agonist/antagonist helps explain a phenomenon recognized for many years: that some ligands act only as partial agonists, capable of binding βAR with high affinity but limited in the activation of adenylate cyclase (AC) (Waldeck et al., 1986, Jasper et al., 1988).

Functional selectivity, potency, and efficacy of agonists may reside in differences in receptor affinity, receptor activation, or both (Mills and Mersmann, 1995). There are

Figure 5 Adrenergic receptor activity

Adrenergic agonists act at either an α or β receptor stimulating a cascade of cellular events. Agonists acting through the β-adrenergic receptor stimulate the G stimulatory protein (G_s) activating the enzyme adenylyl cyclase while those acting through the α-adrenergic receptor inhibit this enzyme.

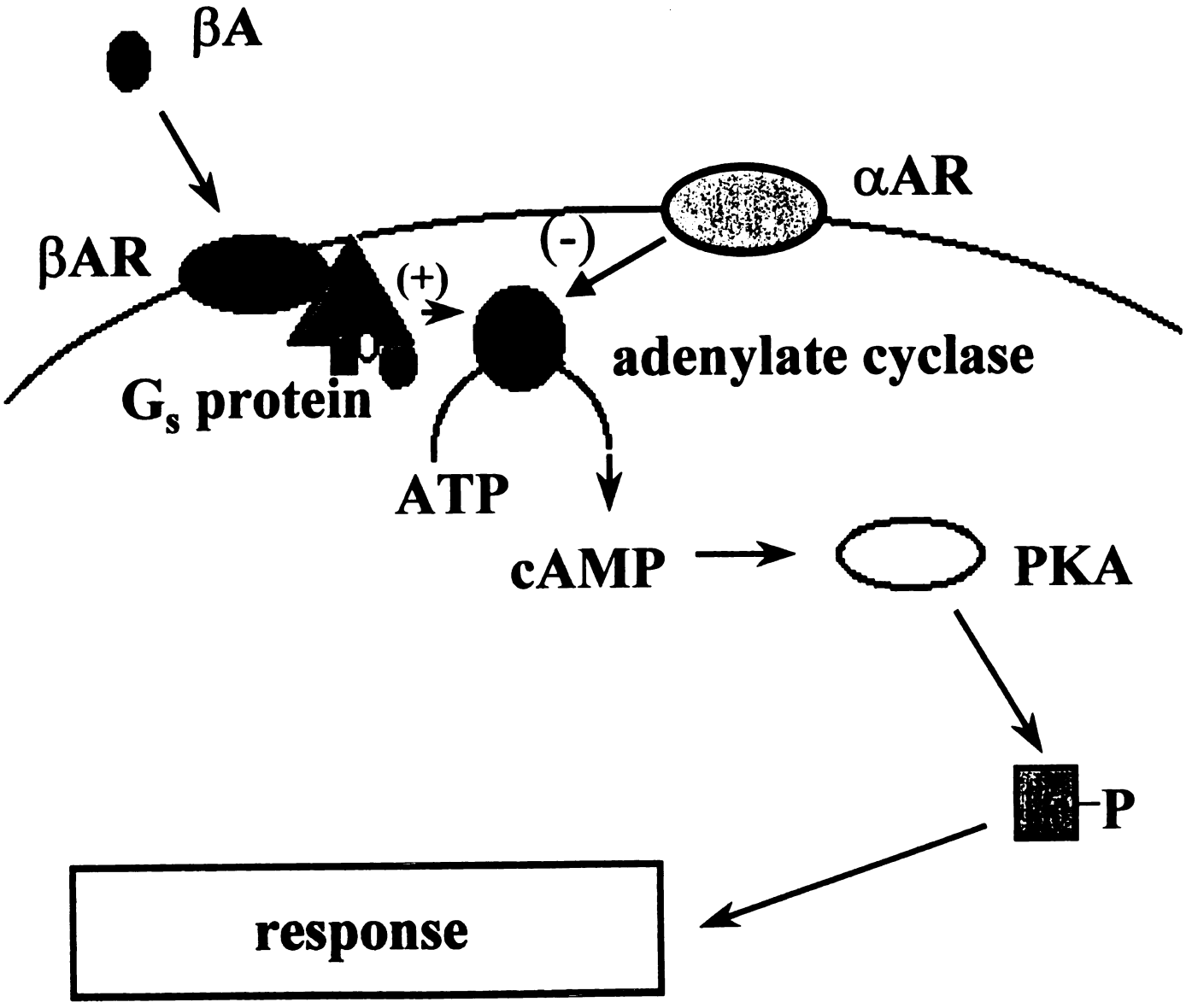
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Figure 6 The β-adrenergic receptor

The structure illustrated represents the human β-adrenergic receptor (Hausdorf et al., 1990). The β-adrenergic receptor is a seven-membrane-spanning domain structure. Intra- and extracellular domains represent regulatory regions. The β-adrenergic agonist binds at the β-adrenergic receptor at extracellular domains activating a cascade of intracelllualr events. Intracellular domains are depicted including key regulatory sites which are substrates for specific kinases involved in β-adrenergic receptor desensitization or turning off the β-adrenergic agonist stimulated signal.

three βAR subtypes, βAR-1, βAR-2, and βAR-3, each representing a separate molecular entity with distinct pharmacological properties (Mersmann, 1998). The βAR's are present on most mammalian cells, but the distribution of subtypes and the proportion of each varies between and within tissues in a given species (Mersmann, 1998). Agonists that bind βAR's activate the enzyme AC, whereas hormones that bind the α-adrenergic receptor inhibit this enzyme. Post-receptor signal transduction is thought to be the same for each of the βAR's (Table 2).

Factors Affecting Responsiveness to β-Adrenergic Agonists

The response to βAA stimulation is dependent on several characteristics of the target tissue. Stimulation at the βAR with βAA results in a profound increase in the intracellular levels of cAMP. Three plasma membrane proteins are known to be required for this effect. The "target" must have functional βAR's, (Moloney, 1991, Lefkowitz et al., 1983, Birnbaumer et al., 1985, Stiles et al., 1984), the stimulatory guanine nucleotide binding protein, and the enzyme AC. The interaction of the βAR complex with the G_s catalyzes the release of GDP from the α subunit of the G protein (α_s), allowing the binding of GTP; this, in turn, leads to the direct activation of AC by α_s-GTP (Levitski, 1988). Upon removal of the agonist, the activation persists until the intrinsic GTPase activity of the α_s hydrolyzes the bound nucleotide (Hausdorff et al., 1990). In addition to the generation of cAMP from AC, two other factors, degradation of cAMP by phosphodiesterases (PDE) and the export of cAMP outside the cell, act to regulate cAMP generation.

Table 2

Adrenoceptor	
α_1	
α_2	
β_1	
β_2	
β_3	

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Table 2 Adrenergic receptor subtypes and post-receptor activity

Adrenergic Receptor Subtype	Post-receptor Activity
α1	Coupled to Protein kinase C, Calcium and IP3
α2	Coupled to Gi, inhibits adenylate cyclase
β1	Coupled to Gs, activates adenylate cyclase
β2	Coupled to Gs, activates adenylate cyclase
β3	Coupled to Gs, activates adenylate cyclase

The subtypes of adrenergic receptors and post-receptor activities are presented. The subtypes are classified as either α or β and depending on the subtype have similar post-receptor activities. Alpha-adrenergic receptors act through a G inhibitory protein (G_i) inhibiting the action of the enzyme adenylyl cyclase (AC). The β-adrenergic receptors act through a G stimulatory protein (G_s) which activates the enzyme adenylyl cyclase and thereby elicits the characteristic cAMP mediated cascade of events.

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The relative distribution of βAR is another example of regulation of the response to βAA stimulation. There is a specific stimulatory G_s protein by which βAR-1 and βAR-2 receptors are coupled to AC in the same manner. In contrast, activation of the α adrenergic receptor, specifically α–2, inhibits AC by promoting the binding of a different G inhibitory protein (G_i) (Birnbaumer et al., 1985). The relative distribution of these receptors may be a means of regulating the biochemical responsiveness of a tissue to adrenergic stimulation (Moloney, 1991).

Regulation of receptor number is yet another factor affecting responsiveness to βAA stimulation (Moloney, 1991). The development of cell receptors and the intracellular mechanism for executing the response to receptor occupancy are a function of cell type and species (Mersmann, 1989).

Another factor affecting receptor number which may represent a more acute control of receptor density is desensitization or down regulation (Stiles et al., 1984, Levitzki, 1986). Prolonged exposure to βAA's results in a decline of receptor numbers at the cell surface and a reduced responsiveness to stimulation (Moloney, 1991). Desensitization may occur through uncoupling of receptor occupancy or through internalization of the receptor at the cell membrane for subsequent recycling and/or degradation (Hoffman, et al., 1979, Stiles et al., 1984, Su et al., 1980, Moloney, A., 1991). Hausdorff et al. (1990) report that the molecular mechanisms behind desensitization do not require internalization of the βAR,

but rather an alteration through ~P by at least two kinases, PKA and βAR-kinase (βARK), which uncouples the receptors from the G_s protein. Phosphorylation sites for each of these kinases are illustrated in Figure 6. It appears that both PKA and βARK are operative simultaneously, although a higher agonist concentration may be necessary to activate βARK (Hausdorf et al., 1990). PKA may be elevated by agents other than βAA (heterologous desensitization) (Mills and Mersmann, 1995). Additional kinases also may be involved in βAR phosphorylation, including protein kinase C (PKC) and an unspecified tyrosine kinase (Lefkowitz et al., 1990, Mills and Mersmann, 1995).

Different βAA have different affinities and, therefore, differing potencies relative to the responses observed (Table 3). Similar compounds may have differing effectiveness and, occasionally, opposite effects in biological systems (Moloney, 1991). Mills and Mersmann (1995) report that two points are important regarding specificity: 1) no agonist or antagonist is absolutely specific for a βAR-1 or βAR-2 because specificity resides in the relative concentration required to stimulate or inhibit the receptor, and 2) the classification system is circuitous in that tissues are classified as having specific receptor subtypes on the basis of their interaction with specific compounds, and compounds are classified as specific for receptors on the basis of their ability to bind with receptors on specific tissues. Differences in the pharmacological properties of agonists and antagonists in different tissues have revealed that multiple tissues express more than one βAR subtype. The potency for activation by common agonists (isoproterenol, epinephrine, and norepinephrine) has been delineated for each receptor subtype.

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Table 3 Adrenergic receptor subtype characterization

	Adrenergic Receptor Subtypes			
	α1	α2	β1	β2
Agonist potencies	Norepi≥Epi>Iso	Norepi≥Epi>Iso	Iso>Epi≥Norepi	Iso>Epi>>Norepi
Antagonist potencies (Subtype selective)	Prazosin>>Yohimbine	Yohimbine>>Prazosin	Metoprolol>>Zinterol	Zinterol>>Metoprolol
Other antagonist	Phentolamine, Phenoxybenzamine	Phentolamine, Phenoxybenzamine	Propranolol, Alprenolol	Propranolol, Alprenolol
Tissue distribution	Vascular Smooth Muscle Hepatocytes	Platelets White Adipocytes	Cardiac muscle	Skeletal muscle Vascular Smooth Muscle Hepatocytes

The table represents adrenergic receptor subtypes and attributed characteristics. The various agonists and antagonists of each receptor subtype are presented relative to their potency. The respective tissues that the receptor sub-types are most prevalent also listed.

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How a cell with multiple βAR subtypes integrates the function of the receptor subtypes remains unclear (Mills and Mersmann, 1995). Despite the apparent similarities in agonist binding in different tissues and activation of AC, other differences have been reported when addressing βAR activation. Whereas PRO completely blocks the activation of AC in adipose tissue, it did not block ISO-stimulated AC in muscle. That PRO did not function as an antagonist in muscle is explained by the fact that this ligand stimulated AC in pig muscle membranes. PRO alone did not stimulate AC activity in adipose tissue membranes, but was as efficacious as ISO in pig skeletal muscle (Spurlock et al., unpublished results). PRO is a potent βAR-1 and βAR-2 antagonist and has been shown to be a partial agonist in the cloned βAR-3 from humans, but not from mice (Blin et al., 1994). The strong agonist response to PRO in pig skeletal muscle clearly distinguishes the βAR in this tissue from those in pig adipose tissue or from tissues in other species examined. Other factors which may influence βAA action include absorption, degradation, excretion, and counter activities by other systems (Mills and Mersmann, 1995).

Manipulation of Animal Growth by β-Adrenergic Agonists

Synthetic βAA are the most potent agents that can promote skeletal muscle growth in many species of animals (Deschaies et al., 1980, Beermann et al., 1986, Wallace et al., 1987, Yang et al., 1989, Sainz et al., 1990, Bergen et al., 1989, Hanrahan et al. 1986). The main advantage with these compounds is that they are orally active and can be incorporated into the diet of food-producing animals. Protein deposition in muscle cells

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in culture can also be enhanced by βAA and this effect can be blocked by β-antagonists (Anderson et al., 1990, Bergen et al., 1991). The fact that the βAA effect can be blocked by a β-antagonist implies that the effect is mediated via the βAA pathway; however, a non- βAR stimulation in skeletal muscle has also been proposed (Bergen et al., 1989, 1991). The mechanism by which βAA influence protein accretion in muscle remains unresolved. The effects may be mediated at a pretranslational level. Skeletal muscle total RNA content, α-actin, myosin light chains 1 and 3 mRNA abundance are increased in animals fed βAA (Smith et al., 1989, Helferich et al., 1990).

The proposed mechanism for βAA-induced muscle protein accretion is through a G_s-protein linked protein kinase cascade (Bergen et al., 1991, Bowman et al., 1969). Most aspects of adrenergic stimulation do not ordinarily require an increase in the synthesis of new proteins, and subsequent events may involve a complex interplay among various proteins by covalent modification through ~ P/dephosphorylation (Iwaki et al., 1990). Protein ~P regulates a diverse range of cellular responses (Meek et al., 1992). Although many of these regulated proteins have been identified and characterized in other systems, many newly discovered or yet undiscovered proteins may play a pivotal role in understanding the biochemical mechanism behind βAA-induced muscle protein accretion (Iwaki et al., 1990, Meek et al., 1992).

Oral administration of synthetic βAA to food-producing or laboratory animals has been shown to increase muscle growth and alter carcass composition to that of a leaner animal

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(Hanrahan et al., 1986, Beermann, 1989, Anderson et al., 1992). Growth rate may or may not be increased, but efficiency is generally improved 10-20% and is primarily the result of the change in composition of gain to a greater percentage of protein and less fat (Mills and Mersmann, 1995). Another characteristic of βAA is increased dressing percentage, indicating carcass tissues are the primary target for βAA action (Mills and Mersmann, 1995). The first reports of βAA manipulation of animal growth centered on the effects of βAA stimulation in lambs (Baker et al., 1984), poultry (Dalrymple et al., 1984), cattle (Ricks et al., 1984), and swine (Dalrymple et al., 1984). The agonists for which the most data are available include ractopamine (RAC), clenbuterol (CLEN), cimaterol, L-644-969, and salbutamol (Mills and Mersmann, 1995). With the exception of RAC, the agonists are reported to have βAR-2 selectivity. Skeletal muscle βAR's have been identified and have been shown to be predominantly βAR-2 (Sainz et al., 1990). Each agonist is not equally effective in each species, and species differences exist in their magnitude of response to the agent tested (Mills and Mersmann, 1995).
Considering postnatal growth of skeletal muscle is primarily the result of hypertrophy and that skeletal muscle DNA is not affected in βAA-treated animals suggests increased protein synthesis. The increase in muscle mass attributed to βAA stimulation may be attributed to an increase in muscle protein synthesis, a decrease in muscle protein degradation, or both (Mersmann, 1998, Beermann et al., 1985, Koomahraie et al., 1991).

Increased rates of protein synthesis have been reported for pigs treated with RAC (Bergen et al., 1989, Helferich et al.,1990) as well as an increase in the amount of RNA transcript

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for several muscle proteins including myosin light chain, SKA (Helferich et al., 1990, Grant et al., 1993, Killefer et al., 1994, Smith et al., 1989). Decreased rates of protein degradation upon βAA treatment are reflected by a decrease in the activity and mRNA abundance for the cathepsins and soluble proteases (calpains) while that of an endogenous protease inhibitor (calpastatin) increased (Mersmann, 1998, Koohmaraie et al., 1991, Higgins et al., 1988, Wang and Beermann, 1988, Bardsley et al., 1992, Killefer et al., 1994).

Mills and Mersman (1995) indicate that considerable interest has focused on the βAR subtypes that mediate increased protein accretion in skeletal muscle. Mills and Mersmann (1995) emphasize that the βAR's that mediate skeletal muscle growth may differ from those that mediate adipose tissue metabolism and this may alter the relative effectiveness of a select βAA to alter body composition.

Effect of β-Adrenergic Agonists on Skeletal Muscle

βAA stimulation via G_s protein results in the elevation of intracellular cAMP levels and subsequent activation of the cAMP-dependent protein kinase (PKA). PKA, a serine/threonine kinase, is capable of ~P a wide array of proteins including nuclear phosphoproteins involved in cell growth. Although the biochemical mechanisms that mediate adrenergic effects are not completely understood, the effects are usually rapid (Iwaki et al., 1990). The effects do not usually require an increase in the synthesis of new proteins. βAA stimulation cascade effects are transmitted through cyclic nucleotide

(Stull et al., 1977, Tsien, 1977, Jones et al., 1986), lipid-derived signaling molecules (Iwaki et al., 1990), and the ~P of critical cell substrates and enzymes (Brostrum et al., 1970).

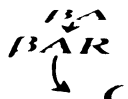
Signal Transduction Pathway Cross-Talk

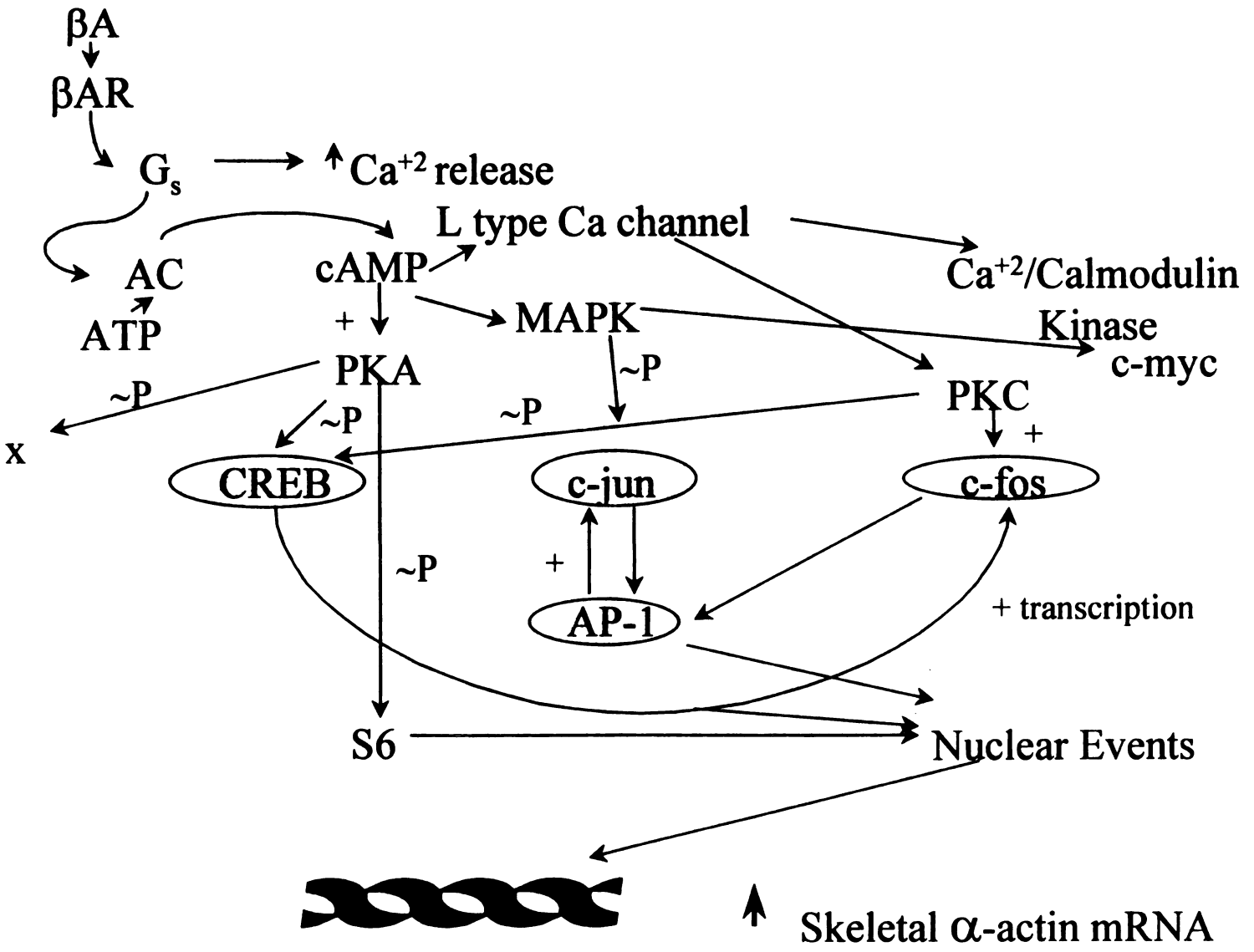
The basic structure of many of the major signaling pathways is well established. Signaling pathways may be composed of a variety of proteins including specific receptors, GTP-binding proteins, second messenger generating enzymes, protein kinases, target functional proteins, and regulatory proteins (Nishizuka, 1992, Barnard, 1992). Interactions across signaling pathways are diverse and include potentiation, cooperation, synergism, antagonism, and co-transmission (Iwaki et al., 1990). The secondary or tertiary messengers that translate the signals into molecular responses at the level of gene expression are not understood and are presumably multifactorial (Iwaki et al., 1990) (Figure 7).

The βAR is coupled to at least two intracellular signal pathways, including cAMP generation (Bishopric et al., 1992, Citri et al.) and Ca^{++} entry (Yatani et al., 1989). In practice, Ca^{++} and cAMP-dependent signaling pathways are closely intertwined, in part because PKA is involved in the regulation of Ca^{++} homeostasis at many intracellular sites, and both pathways may converge on the same transcriptional activator (Bishopric et al., 1992, Sheng et al., 1990). Ca^{++} and cAMP have been implicated in eukaryotic gene-regulatory pathways (Bishopric et al., 1992, Roesler et al., 1988). The cell surface

Figure 7 Schematic of signal transduction pathways and possible cross-talk interactions

A complex interplay among signal transduction pathways and key regulatory intermediates may influence skeletal α-actin gene transcription. Key signaling transduction cascades and their pathway component modulators are represented. These cascades may interact at specific trans-factors initiating gene expression.

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receptor-mediated generation of second messenger molecules, such as cAMP and Ca^{++}, and the subsequent activation of protein kinases and phosphatases is a widespread mechanism of signal transduction in mammalian cells (Rozengurt, 1986). Many transcription factors are phosphoproteins and their functions could, therefore, be regulated by phosphorylation/dephosphorylation events (Mitchell et al., 1989).

The pathways leading from cAMP and PKA are incompletely understood. Phosphorylation of 40S ribosomal protein S6 is elicited by agents, including cAMP and PKA, in a wide variety of tissues which correlates with increased rates of translation (Wettenhall et al., 1982, 1984, Gressner et al., 1980, Stefanovic et al., 1986). Although PKA can directly ~P S6, there are also two kinase families: the 70 kDa and 90 kDa kinase families (Sturgill et al., 1991, Erikson, 1991). The signaling cascade regulating the 70 kDa family is poorly understood but it can be inhibited by rapamycin and stimulated by cAMP (Chung et al., 1992, Kahan et al., 1992). In contrast, the 90 kDa family is ~P and activated by mitogen-activated protein kinase, MAP kinase (Sturgill et al., 1990, Chung et al., 1991). Furthermore, upon stimulation, MAP kinase has been shown to translocate from the cytoplasm to the nucleus and influence transcriptional events through regulation of factors such as c-myc, c-fos, c-jun and ATF-2 (Davis, 1993).

In L_6 cells, $COS\text{-}_7$ cells and PC_{12} cells, cAMP has been shown to activate MAP kinase (Davis, 1993, Faure et al., 1994, Frodin et al., 1994, Thompson et al. 1996). Both PKA and elevated levels of cAMP have been shown to stimulate ribosomal protein S6

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phosphorylation which may promote the recruitment of mRNA to increase the number of polysomes (Wettenhall et al., 1982,1984, Gressner et al., 1980, Chung et al., 1992). Is it possible that cAMP may alter translation and/or transcription rates through a mechanism involving MAP kinase in differentiated C_2C_{12} cells?

In mammalian cells, the ability to activate the mitogen-activated protein (MAP) kinase cascade is a feature common to many extracellular stimuli including growth factors, hormones, and neurotransmitters (Pelech et al., 1992, Crews et al., 1992). The various stimuli which can activate the MAP kinase cascade employ distinct initial signaling pathways (Frodin et al., 1994). In the myocyte, the βA, ISO, stimulates protein synthesis and mimics other hypertrophic agents in activating MAPK (Bogoyevitch et al., 1996, Mills, 1998). Activation of MAPK is Ca^{++} dependent but not cAMP dependent, suggesting the involvement of multiple G proteins in βAR signaling (Mills, 1998). In HEK293 cells, isoproterenol activates MAPK via the βγ subunit of G_i. Only the βARK ~P receptor activates MAPK suggesting desensitization of the G_s pathway is the signal to activate the G_i pathway (Daaka et al., 1997, Mills, 1998) however, at the same time, MAPK activation in the myocytes is pertussis toxin insensitive, indicating multiple pathways to MAPK activation are possible (Mills, 1998). Some stimuli activate receptor tyrosine kinases or non-receptor tyrosine kinases (Klausner et al., 1991, Gupta et al., 1993, Gardner et al., 1993, Ahn et al., 1991, Sturgill et al., 1988, Boulton et al., 1991). Other stimuli activate G protein-coupled receptors generating second messengers, including diacylglycerol (DAG) or Ca^{++}, or activating ion channels (Bading et al., 1991,

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Ely et al., 1990, Vournet-Craviari et al., 1993, Winitz et al., 1993). Frodin et al. (1993) report that cAMP activates the MAPK cascade in PC_{12} cells; the significance here lies in the fact that the data indicates a cAMP/MAPK cascade link (Frodin et al., 1994). Frodin and co-workers (1994) show a cAMP effect and a synergistic effect between cAMP and phorbol ester (PE), which activates PKC. Such an interaction may be used by different ligands that stimulate cAMP generation or phosphatidylinositol breakdown, respectively, or by ligands that stimulate both pathways.

Various studies have suggested a requirement for intracellular Ca^{++} in βAR-mediated actions (Horn et al., 1988, Putney, 1978, Argent et al., 1985). βAR activation has also been reported to directly alter Ca^{++} flux mechanisms in parotid acinar cells (Horn et al., 1988, Kanagasuntheram et al., 1976, Butcher, 1980, Scott et al., 1985, Takemura, 1985, Dreux et al., 1986, Helman et al., 1986, Nauntofte et al., 1987); however, these intracellular events have not been characterized (Horn et al., 1988). Horn et al. (1988) report that in rat parotid cells, there is an interaction between the cAMP and phosphoinositide intracellular signaling systems. Horn et al. (1988) report that ISO stimulates PIP-2 turnover and mobilizes Ca^{++} from an intracellular, carbachol-sensitive Ca^{++} pool via a mechanism involving βAR and cAMP. βAR's activate the G protein, G_s, which stimulates Ca^{++} currents by both cytoplasmic, indirect, and membrane-delimited direct pathways (Yatani et al., 1989, Gilman , 1987, Yatani et al., 1987, Brown et al., 1988). The βAA, ISO, increases Ca^{++} currents through a cAMP pathway (Yatani et al., 1989, Trautwein, et al., 1987). It has been shown in single-channel patch clamp studies

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that the βAR and G_s are directly coupled to the L-channel (Yatani et al., 1989). In theory, signal mechanisms involving Ca^{++} entry through the myocardial L channel should be activated by any agent that increases intracellular cAMP, since the channel is ~P and activated by PKA (Reuter et al., 1982), and this activation accounts for the bulk of measurable βAR stimulated Ca^{++} entry (Yatani et al., 1988, Hartzell et al., 1991). Bishopric et al. (1992) report that cAMP and forskolin (FOR) had relatively minor effects on SKA expression as compared to ISO and cholera toxin (CTOX). Bishopric (1992) suggests that βAA induction of SKA may require participation of a CTOX-sensitive G_s protein which would be bypassed by FOR and cAMP. They have also shown that inactivating PKA has no effect on the upregulation of SKA by ISO. They report that in cardiac myocytes, downstream elements of the cAMP/PKA pathway are not required.

Transcriptional Regulation by Extracellular Signals Through Phosphorylation

Changes in cellular gene transcription patterns induced by extracellular signals are thought to be important for many biological processes (Bohmann, 1990). The transmission of gene regulatory signals through the cytoplasm is mediated by signaling pathways, of which protein kinases are important components. Recent evidence suggests that communication between the cytoplasm and the nucleus relies on signal-dependent ~P/dephosphorylation of transcription factors (Bohmann, 1990). To activate or repress transcription, transcription factors must be located in the nucleus, bind DNA, and interact with the basal transcription apparatus. Accordingly, extracellular signals that regulate transcription factor activity may affect one or more of these processes. Most commonly,

regulation is achieved by reversible ~P. Phosphorylation of a transcription factor by several different kinases, or a kinase linked to more than one pathway, is a simple mechanism that allows different signals to converge at the same factor. Many growth-factor induced genes themselves encode transcription factors, and it is presumably the interaction between these factors and pre-existing factors, including those activated by the stimulus itself, that ultimately determines the response of the cell to the stimulus (Hill et al., 1995). Given the potential complexity of such interactions, it is conceivable that even if two different stimuli induce the same set of genes, small differences in relative expression might result in qualitatively different patterns of subsequent transcription.

The growing list of such nuclear effector proteins include the transcription factors, CREB, Jun, Fos, NFKB, Myc and Myb. Four of which (Jun, Fos, Myc, and Myb) are products of proto-oncogenes, and two tumor suppressor proteins, p53 and the product of the retinoblastoma susceptibility gene (pRB), and the simian virus 40 (SV 40) large tumor antigen (T antigen) which is a multifunctional viral protein involved in DNA replication, transcription and cellular transformation (Meek et al., 1992). Several of these proteins are ~P by the same protein kinases, suggesting that signals may coordinately target these key proteins (Meek et al., 1992). Although it is clear that βAR stimulation must generate intracellular mediators that reach the nucleus and consequently activate skeletal muscle-specific gene expression, the precise signaling mechanisms that link the occupancy of the receptor with gene induction remain unknown (Iwaki et al., 1990).

Expression of the proto-oncogenes c-fos and c-jun is induced by a wide variety of agents. such as mitogens, differentiation factors, specific pharmacological agents, stress, and heat shock (Greenberg et al., 1984, Kruijer et al., 1984, Muller et al., 1984, Lamph et al., 1988, Ryder et al., 1988, Ransone et al., 1990). Induction is rapid and transient and occurs at the level of transcription (Ransone et al., 1990) and the regulation of c-fos and c-jun expression is via not only their gene products, but also by related transcription factors (Rasone et al., 1990).

Fos

According to its putative role as a master switch in cell proliferation and differentiation, c-fos transcription is increased in response to various stimuli. Depending on the cell line, these stimuli include growth factors and cytokines like PDGF, FGF, EGF, NGF, TNFα, TGFβ, thyrotropic hormone, PE, Ca^{++} ionophore, metal ions, neurotransmitters, heat shock, cAMP, and UV irradiation. Expression of the fos gene appears within minutes of induction, reaches maximal levels by 30-60 minutes and is essentially undetectable by 120 minutes (Ransone et al., 1990). The rapid induction is likely to involve post-translational modifications of the fos protein (Ransone et al., 1990). In the presence of inhibitors of protein synthesis, the c-fos mRNA is induced at the normal rate, but remains detectable for up to 4-6 hours (Ransone et al., 1990). This suggests that, following mitogen treatment, pre-existing factors are utilized for fos transcriptional activation (Treisman, 1986, Sassone-Corsi et al., 1987). The c-fos protein can repress the

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transcription of the c-fos gene (Lucibello et al., 1989, Sassone-Corsi 1988c, Schonthal et al., 1988, Wilson et al., 1988).

As mentioned earlier, the induction of the fos gene may be carried out through the mediation of adenylate cyclase and PKC pathways, two major signal transduction pathways in the cell (Ransone et al., 1990). Although multiple protein-binding sites within the c-fos promoter were identified, for most of the inducers listed above the respective cis-acting element that mediates the effect has yet to be determined (Angel et al., 1991, Verma et al., 1987). Inspection of the c-fos promoter reveals the presence of a 20 bp region required for responsiveness to serum and growth factors and is identified as a palindromic sequence known as the dyad symmetry element at approximately -308 bp (Angel et al., 1991). Further investigation reveals the presence of two cAMP-dependent response elements at positions -60 and -350 required for induction by agonists of the AC pathway (Sassone-Corsi et al., 1988, Gilman et al., 1986). Induction of the c-fos gene requires either activation of the serum response factor or ~P of CREB protein by a catalytic subunit of PKA (Ransone et al., 1990). The c-fos protein represents an excellent example of regulation across signal transduction pathways.

Jun

The proto-oncogene, c-jun, is a component of the AP-1 transcription factor family involved in the mediation of nuclear events elicited by extracellular stimuli (Bohman et al., 1987, Angel et al., 1988, Woodgett, 1990, Pulverer et al., 1991). In contrast to the

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extensive delineation of the regulatory elements in the c-fos promoter, little is known about the c-jun gene. Agents which induce the c-fos gene generally induce c-jun transcription (Ransone et al., 1990). The c-jun promoter has an AP-1 binding site that is recognized by the fos/jun complex (Ransone et al., 1990). Unlike the fos gene, the c-jun gene is positively regulated by its own gene product (Ransone et al., 1990, Angel et al., 1988, Lamph et al., 1990). The c-jun protein is negatively regulated by ~P of residues at the carboxy terminus which are de~P in response to PE (Binetruy et al., 1991, Pulverer et al., 1991) and CREB protein (Lamph et al., 1990). Suppression of c-jun by CREB can be overcome by ~P of CREB with the catalytic subunit of PKA (Lamph et al., 1990). Interestingly, purified c-jun is not a substrate for either PKC or PKA, indicating that PKC activation does not directly lead to modification of the c-jun protein (Hai et al., 1988, Angel et al., 1991). A further explanation of the regulation of the c-jun gene extends beyond the scope of this literature review. Suffice to say that c-jun is regulated by the same elements which regulate c-fos expression with the main difference being that the c-jun protein elicits positive feedback on its own expression.

Activator Protein-1

Activator protein-1, AP-1, is the collective name for a class of transcription factors that is functionally characterized by the ability to bind to promoter or enhancer elements containing TGACTCA or related sequences (Bohmann, 1990). The members of the AP-1 family of transcription factors include c-jun, jun-B, jun-D, c-fos, Fos B, Fra-1, and Fra-2 (Bohmann, 1990). Structurally, all these proteins share a conserved region consisting of

the leucine repeat dimerization domain (leucine zipper) and an adjacent basic DNA binding domain (Bohmann, 1990). The functional form of AP-1 is a dimer that is composed of either two jun monomers, or of one jun and one fos or fra monomer (Bohmann, 1990). AP-1 was first identified as a transcriptional factor that binds to an essential cis-element of the human metallothionein II promoter and has also been recognized as a TPA response element of several genes whose transcription is induced in response to treatment of the cells with a PE tumor promoter (Karin et al., 1991, Huang et al 1991). Evidence suggesting that protein ~P is involved in the regulation of AP-1 activity came from the observation that the positive effect of AP-1 binding sites on the transcription of linked genes could be increased by exposure of cells to TPA (Bohmann, 1990, Angel et al., 1987, Lee et al., 1987). TPA is a potent activator of PKC (Nishizuka, 1984). Other agents which lead to PKC activation, such as serum, and growth factors, also induce expression of these genes (Imbra et al., 1986, Imbra et al., 1987 Angel et al., 1987, Brenner et al., 1989, Matrisian et al., 1986). Inhibitors of PKC block those responses (Imbra et al., 1987 Angel et al., 1987, Brenner et al., 1989). Comparison of several transcription response elements led to the derivation of a palindromic consensus sequence that is recognized by AP-1 : 5'-TGA G/C TCA-3' (Angel et al., 1987, Lee et al., 1987). Insertion of synthetic oligodeoxynucleotides that form efficient AP-1 binding sites in front of the thymidine kinase promoter renders it TPA inducible (Karin et al., 1991). Sequences that deviate from the consensus in positions essential for AP-1 binding do not confer such a response (Angel et al., 1987). Hence, AP-1 binding is sufficient for conferring a transcriptional response to PKC activation (Karin et al., 1991).

Cyclic AMP Response Element Binding Protein

Cyclic AMP response element binding protein (CREB) was initially identified as an activity that could bind to cAMP-responsive promoter elements (CRE's) (Bohmann, 1990). The situation with CREB is different than that of AP-1 in that a clear connection has been discovered between an inducer (cAMP) , a kinase (protein kinase A), a ~P site (ser-133) and a transcriptional effect (Bohmann, 1990). cAMP activates PKA, which is capable of entering the nucleus and ~P CREB, the cAMP response element binding protein, on ser 133 (Gonzalez et al., 1989). The CREB~P is then capable of binding to the CRE, cAMP response element, and enhances transcription of linked promoters (Boularand et al., 1995, Kinane et al., 1993, Rangan et al., 1996).

Iwaki and coworkers first demonstrated that the α or β adrenergic stimulation increased induction of the immediate early genes c-fos and c-jun. Since these genes encode either known or putative transcriptional factors, their induction has been proposed to regulate gene transcription during growth factor stimulation, thereby influencing cellular growth and/or differentiation (Iwaki et al., 1990). Previous research by Bishopric and coworkers (Bishopric et al., 1992 a,b) has indicated a direct relationship between specific transcription factor activation (c-fos, c-jun) and signal transduction pathway cross-talk in the regulation of SKA gene expression in cardiac myocytes. The regulation of SKA gene expression in skeletal muscle may very well involve such a network of communication. Figure 1 represents a diagram compiled from literature in different cell types across

signaling pathways. The diagram represents the possible complexity that may exist in trans-factor regulation of gene expression across signal transduction pathways.

Influence of Phosphoproteins on Skeletal α-actin Transcription

Early work by Bishopric et al.(1992) indicated that norepinephrine (NE) induces both hypertrophy and SKA gene expression in cultured neonatal rat myocytes (Bishopric et al., 1992, Bishopric et al., 1992). The response of cardiac myocytes to NE involves one or both α and β-adrenergic signal pathways (Bishopric et al., 1992, Simpson et al., 1982, 1985). In confluent myocytes, SKA induction required only the βAA component and could be reproduced by the βAA, ISO, instead of NE (Bishopric et al., 1992). Bishopric et al. (1992) reported that expression of the proto-oncogenes c-fos and c-jun is activated very early in the hypertrophy response of myocytes to ISO, preceding SKA expression by several hours (Bishopric et al., 1992). Overexpression of transfected c-fos and c-jun in cardiac myocytes and in P_{19} teratocarcinoma cells strongly and selectively activated the SKA promoter in the absence of serum or other growth stimuli (Bishopric et al., 1992). The data presented by Bishopric et al. (1992) support a possible functional relationship between early proto-oncogene expression and SKA induction during signal-mediated hypertrophy of rat cardiac myocytes (Bishopric et al., 1992).

Analysis of the SKA promoter did not reveal a canonical AP-1 consensus element (Bishopric et al., 1992); however, there is evidence for binding of AP-1 to non-consensus sequences (Owen et al., 1990, Takimoto et al., 1989, Gaub et al., 1990, Bishopric et al.,

1992) and there are several elements in the proximal promoter region with partial homology to the AP-1 consensus (TCACTCA) (Taylor et al., 1988, Bishopric et al. 1992). One of the sequences overlaps with the first CArG box, which has been implicated in muscle-specific expression of the α-actins (Minty et al., 1987, Miwa et al., 1987 a,b), and is a binding site for myocardiocyte nuclear proteins within the -153 to -36 region of the SKA promoter.

My dissertation research focuses on the mechanism regulating the βAA-stimulated increase in SKA mRNA abundance in skeletal muscle. I will focus on the signal transduction pathway components and their involvement in transcriptional regulation of SKA using a SKA isoform specific DNA probe. Further insight on the regulation of SKA mRNA abundance following βAA stimulation will be obtained by examining the ~P status of proteins in cell extracts. Phosphorylation regulates a wide array of processes in the cell including trans-factor activation. Trans-factor activation is directly involved in the regulation of gene transcription. Information regarding the mechanism behind βAA stimulation of SKA mRNA abundance obtained through examination of pathway modulation may be beneficial in the optimization of this particular repartitioning strategy.

CHAPTER 1

DEVELOPMENT AND CHARACTERIZATION OF A SKELETAL α-ACTIN SPECIFIC DNA PROBE

Abstract

A DNA probe homologous to 105 bp of the 3' untranslated region of the mouse SKA gene corresponding to bp 3591 to 3696 was prepared by the PCR using a "touch-down" procedure. The PCR fragment was sequenced on a 6% acrylamide gel using the USB Sequenase kit as per manufacturer's instructions. The sequence was verified by comparison to the catalogued sequence of mouse SKA and corresponds to 3591 bp to 3696 bp. The amplified fragment was purified and size verified on a 1.2% agarose gel. Purification of the fragment from the agarose gel was accomplished using the Bio-Rad Prep-A-Gene kit as per manufacturer's instructions. The isolated and purified fragment was resuspended and stored in sterile milli-Q water. In order to determine the usefulness of the probe in agriculturally important species, the probe was tested against total RNA across various livestock species and isoform specificity across various tissue types. Species and tissues tested included mouse, rat, chicken, porcine, ovine, and bovine brain, heart, skeletal muscle, stomach, and liver. Results indicate specificity to the SKA isoform in mammalian species. No activity was detected in any chicken tissues or non-skeletal muscle tissues in the mammalian species tested.

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Introduction

Skeletal muscle is comprised of a variety of proteins. The contractile apparatus is composed of two of the more predominant proteins in muscle, actin and myosin. Actin is unique in that it is a member of a large group of structurally related isoproteins which are also among the most abundant proteins in eukaryotic cells. In addition to their major role in muscle contraction, the actins are involved in maintenance of cell structure and organelle motility, including cytoplasmic streaming, phagocytosis, and cell division (Vandekerckhove et al., 1986, Mayer et al., 1984, Garner et al., 1989). At least six different vertebrate actin isoforms have been identified, each a product of a different gene expressed in different cells or even in the same cell at different stages of development (Vandekerckhove et al., 1986, Garner et al., 1989, Minty et al., 1981). The six actins identified include SKA, CAA, aorta smooth muscle α-actin, smooth muscle α-actin, and two cytoplasmic actins β– and γ-actin. These isoforms can be separated by isoelectric focusing, although they are greater than 90% homologous in amino acid sequence (Minty et al., 1981). Amino acid sequence analyses, confirmed and extended by the DNA sequences of different actin genes have failed to reveal amino acid differences between the same isoactins in different species (Vandekerckhove et al., 1986). The six different isoactins can be classified according to the tissues in which they appear as the major

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forms (Vandekerckhove et al., 1986). Two isoforms are present in the cytoplasmic microfilaments of most or all cell types, while other actin isoforms are found in the contractile apparatus of skeletal, cardiac, and smooth muscle (Mayer et al., 1984, Minty et al., 1981). Non-muscle cells express two homologous isoactins generally referred to as β and γ-cytoplasmic actins (Vandekerckhove et al., 1986). Smooth muscle tissues express two closely related isoforms, referred to as γ smooth muscle and α smooth muscle actin (Vandekerckhove et al., 1986). The γ type is the major form in visceral tissue, while the α type appears as the major form in vascular smooth muscle (Vandekerckhove et al., 1986). In smooth muscle, the γ and α types differ in about 20-23 residues from the non-muscle variants and are similar to, but distinct from, the major isoforms expressed in striated muscle (Vandekerkhove et al., 1986). Cardiac and skeletal striated muscles contain characteristic actin isoforms which have identical molecular weights and isoelectric points, and only the complete amino acid sequence has revealed differences, specifically in the rat heart ventricle (Vandekerkhove et al., 1986). No fiber-type-specific isoforms of actin have been found.

The skeletal muscle type α skeletal actin differs from the cardiac variant by a Glu to Asp exchange at residues 2 and 3 and by exchanges of Met to Leu and Ser to Thr at positions 299 and 358, respectively. The two latter exchanges are also typical of smooth and non-muscle actins (Vandekerkhove et al., 1986). Pairs of these genes are differentially expressed throughout development and in adult tissues (Garner et al., 1989). Two sarcomeric actins, CAA and SKA have been defined (Garner et al., 1989). They are

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coexpressed at high levels during the development of striated muscle but in the adult, the CAA and SKA isoforms predominate in cardiac and skeletal muscles, respectively (Garner et al., 1989). Both actin isoforms migrate at 1.6 kb on an agarose gel.

Skeletal α-actin

Skeletal α-actin represents a highly conserved muscle-specific protein in skeletal muscle. The fact that SKA is (1) muscle specific and (2) is not characterized by developmental and fiber type isoforms, make SKA a unique candidate for studying muscle growth. Actin is the second most abundant contractile protein in skeletal muscle myofibrils and represents 22% of total myofibrillar protein (Yates and Greaser, 1983). The skeletal muscle isoform, skeletal α-actin, represents greater than 95% of all actin present in mature skeletal muscle. The inherent problem with using skeletal α-actin as an index of myofibrillar gene expression lies in the highly conserved nucleic acid sequence among the actin family of genes. Nucleic acid probes for actin tend to cross react among actin isoforms, making data difficult to interpret. Segments of the 3'UTR are similar across species and other sequences may be not only isoform specific but also species specific (Gunning et al., 1984, Skjaerlund, 1993). The amino acid sequences of the different actins are highly conserved whereas the 3' UTR of some actin mRNAs have diverged considerably in their nucleotide sequence. The 3'UTR shows the greatest diversity across isoforms, even though select sequences are conserved across species (Gunning et al., 1984). The 3' UTR of many genes may confer specificity across isoforms (Mayer et al., 1984, Minty et al., 1981, Wettenhall et al., 1982).

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The objective of the initial study was to isolate and characterize a SKA-specific PCR-generated probe homologous to a region of the 3' UTR of the SKA gene. The isoform-specific probe will be useful for experimental analysis of the nuclear events preceding translation of SKA mRNA.

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Materials and Methods

Plasmid

Inconsistencies in the pMACT-α (Hu et al., 1986) plasmid map required verification of sequences before initiation of experiments. A 931 bp Bam HI fragment corresponding to bp 3071-4002 of the mouse SKA gene in pMACT-α was subcloned into the plasmid pcDNA3 (Stratagene, Inc., La Jolla, CA) for sequence verification. Following the sequence verification of the 931 bp fragment, a 437 bp Pst-I/BamHI fragment corresponding to bp 3565-4002 of the mouse SKA gene was subcloned into pBluescript II SK+/- (Stratagene, Inc., La Jolla, CA). The intentions were to allow for maximal replication by PCR of the 437 bp fragment to be collected by plasmid preparation for use as a PCR template. Bacteria were transformed and screened on X-gal plates as per established protocols (Maniatis et al., 1989). Plasmid preps were performed on selected colonies. BamHI/Pst-I digests of the p451 were run on a 1% agarose gel, size verified by comparison to a 100 bp ladder (Gibco, Grand Island, NY), excised and purified using the Prep-A-Gene kit (Bio-Rad, Hercules, CA). A set of appropriate primers was chosen for PCR amplification of a 105 bp region within the 3' UTR of the SKA gene using a "touch-down" procedure. Initially, the 437 bp Pst-1/Bam HI fragment was used as template for amplification of a 105 bp region of the 3'UTR. Subseqently; the 105 bp amplified and purified fragment was used as template for PCR in further amplification reactions.

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Primers

Primers were chosen according to accepted protocol (Maniatis et al., 1982). The primers were prepared at the Michigan State University Macromolecular Structure Facility. The oligonucleotides:

WGB1 (5' TTG GAG CAA AAC AGA ATG GCT G 3') and

WGB2 (5'ATA GAT TGA CTC GTT TTA CCT CA 3')

were chosen as primers for the polymerase chain reaction and represented sequences complementary to bp 3674-3696 and 3591-3612 bp of the mouse SKA gene, respectively.

Polymerase Chain Reaction

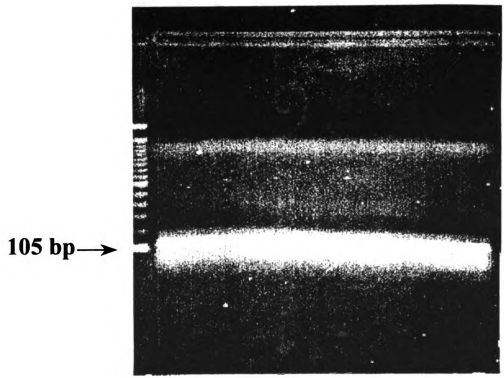
A digoxygenin-labeled probe was created using the Boehringer Mannheim Biochemicals-Digoxygenin PCR Kit (BMB, Indianapolis, IN) using the Bam HI/Pst-I 437 bp fragment as the initial template and the oligonucleotide primers WGB 1/2 in a "touch-down" procedure. The amplified fragment was size verified using a 100 bp DNA ladder (Gibco, Grand Island, NY) at 105 bp (Figure 8) and purified from an agarose gel using the Prep-A-Gene kit from Bio-Rad. The "touch-down" procedure allows for efficient amplification of templates which have higher calculated annealing temperatures (>50) (see Appendix A).

Sequencing

A 931 bp Bam HI fragment subcloned in the initial plasmid clone pS3'α of pcDNA3 was sequenced at the Michigan State University Plant Research Laboratory using the

Figure 8 Skeletal α-actin 105 bp PCR product

The figure depicts a 1.2% agarose preparative gel and the product of the skeletal α-actin PCR "touch-down" reaction. The PCR product represents a 105 bp region of 3' untranslated region of the mouse skeletal α-actin gene. The product is size was verified at 105 bp by comparison to a 100 bp molecular weight marker.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Dyedeoxy procedure from the T7 dye primer or Sp6 dye primer, a system designed by Applied Biosystems Inc. The Dyedeoxy method of sequencing is similar to the Sanger dideoxy method which represents a controlled interruption of enzymatic replication. The Dyedeoxy chromatogram is pictured in Appendix B.

A 437 bp Bam HI/Pst-1 fragment was subcloned into pBluescript for further verification of the proper α-actin sequence and is intended to be the initial template for the PCR of the 105 bp fragment. The 437 bp fragment was sequenced using the Sequenase protocol (United States Biochemical (USB), Cleveland, OH) from the T7 and T3 promoters and verified by comparison to the published sequence of mouse SKA on GenBank (Name: MUSACASA, Accession: M12347) to be homologous to 3565 bp through 4002 bp of the mouse SKA gene (see Appendix C).

The 105 bp fragment obtained from the PCR reaction using the Bam HI/Pst-I 451 bp fragment of the pBluescript plasmid clone and WGB1/2 primers was sequenced using the Sequenase protocol (USB) and verified by comparison to the published sequence of mouse SKA on GenBank (Name: MUSACASA, Accession: M12347) to be homologous to 3591 bp through 3696 bp of the mouse SKA gene (Figure 9).

RNA Isolation

Total RNA was obtained from distinct tissues across species: bovine, ovine, porcine, rat, mouse, and chicken. Tissues were chosen to represent those tissues predominantly

Figure 9 **Sequence analysis of a PCR generated 105 bp fragment of a 3' untranslated region of the mouse skeletal α-actin gene**

The text represents the nucleotide sequence of a 105 bp region of the mouse skeletal α-actin gene 3' untranslated region. Sequence analysis confirmed the sequence of the 105 bp PCR product to be identical to the mouse skeletal α-actin 3' untranslated region from 3591 bp to 3696 bp. Verification was accomplished through a homology match to the published sequence of the mouse skeletal α-actin gene on a genomic database. Primer locations are underlined and designated as either WGB1 or WGB2.

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5' **wgb2** **3'**

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AACTTGAAGCATTAAAGCCAGCCATTCTGTTTTGCTCCAA

5' **wgb1** **3'**

3674 **3696**

expressing the non-skeletal muscle actin isoforms present in various species tissues. Tissues tested included brain, heart, liver, stomach, and skeletal muscle. The "stomach" samples for ruminant and avian species were abomasum and gizzard, respectively. Total RNA was isolated from 1 g tissue samples using 10 ml Trizol reagent (GIBCO BRL. Co., Grand Island, NY). Tissue samples were homogenized with a Kinematic polytron homogenizer (model # PT 10/35) at setting #4 for approximately 30 seconds. Following homogenization, the sample remained at room temperature for fifteen minutes to allow for the complete dissociation of nucleoprotein complexes before an initial pre-spin at 12,000 x g for 5 minutes at 4° C. The supernatant was then extracted against chloroform (.2 ml chloroform per 1 ml Trizol reagent, Gibco, Inc., Grand Island, NY) and phase separation achieved by centrifugation at 12,000 x g for 15 minutes at 4° C. The RNA was precipitated from the extract with isopropanol (.5 ml per 1 ml Trizol reagent), stored on ice for 10 minutes and followed by centrifugation at 12,000 x g for 20 minutes at 4° C. The RNA pellet was then washed by vortexing with 75% ethanol (1 ml per 1 ml Trizol reagent) and centrifuged at 7,500 x g for 5 minutes at 4° C. The RNA pellet was resuspended in sterile milli-Q diethylpyrocarbonate (DEPC) treated RNase free water and stored at -80°C until analysis. RNA solutions were scanned spectrophotometrically from 220 nm - 320 nm, the A260/A280 ratio was determined to check quality of preparation, and RNA concentration calculated from the A260.

Northern Blot Analysis

Ten micrograms total RNA extracted from tissue samples was electrophoretically separated on a denaturing 1.2% agarose, 2.2 M formaldehyde gel at ~60 V for 3 hours. A control RNA, isolated from mouse hind-limb muscle, was used in all studies to aid in the normalization of hybridization data across studies. RNA was transferred to positively charged nylon membranes (Boehringer Mannheim Biochemicals, Indianapolis, IN) overnight with a 10x SSC solution. Following Northern transfer, the membranes were allowed to dry slightly and were then UV crosslinked using a Spectroline transilluminator (model # TR-302) for three minutes before prehybridization in a standard hybridization buffer (5X SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, and 1% blocking reagent) for 8 hours. Blots were hybridized with a digoxygenin labeled 105 bp PCR generated skeletal α-actin probe at 42° C for 8 hours. After hybridization, membranes were washed (2 x 5 minutes) in a 2x SSC/ .1% SDS solution at 42° C and then (2 x 15 minutes) in a .1x SSC/ .1% SDS solution at 42° C. Membranes were blocked in a maleic acid buffer (0.1M maleic acid, 0.15 M NaCl, pH 7.5 and 10% w/v blocking reagent) for 30 minutes and then exposed to anti-Dig Fab fragments (Boehringer Mannheim Biochemicals, Indianapolis, IN) for 30minutes. Membranes were washed (2 x 15 minutes) in a .3% Tween-20 Maleic acid buffer, rinsed in a Tris buffer (100 mM Tris-HCl, pH 9.5, 100mM NaCl) for one minute and exposed to the chemilluminescent alkaline phosphatase detection substrate, CDP-*Star* (Boehringer Mannheim Biochemicals, Indianapolis, IN), for five minutes, sealed in plastic bags, and exposed to x-ray film. Hybridization of RNA

to the 105 bp SKA probe was quantified by videodensitometry. Size was verified at 1.6 kb using an RNA marker (Promega Co., Madison, WI).

Results and Discussion

Skeletal α-Actin Isoform Specificity

It has been suggested that the 3'UTR of the actins may confer isoform specificity over coding regions used for preparation of DNA probes to the actins (Cleveland et al., 1980, Minty et al., 1981, Ponte et al., 1984). Probes prepared from the 3'UTR may be able to distinguish between the α-actin isoforms and allow for a more precise examination of specific α-actin isoform mRNA. Initially, the 105 bp probe was hybridized with mouse hind limb muscle total RNA. Through comparison to an RNA marker (Promega, Inc. Madison, Wisconsin), it was confirmed that the 105 bp probe hybridized to a band at ~1.6 kb, indicating that the probe did hybridize to a transcript consistent with the molecular weight of SKA in mouse hind limb skeletal muscle (Figure 10).

Species and Actin Isoform Specificity

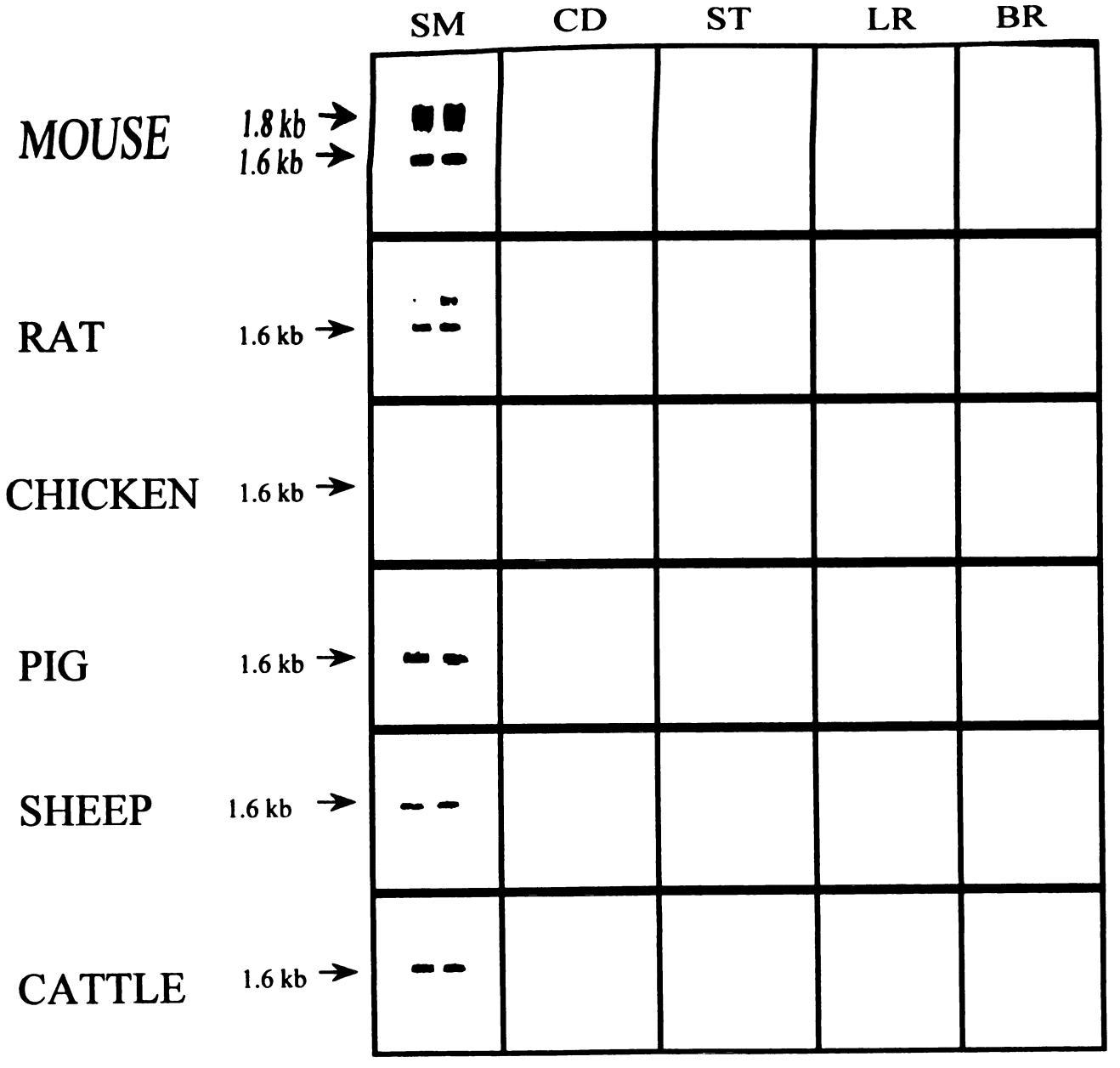
In order to examine the usefulness across species, the 105 bp probe was hybridized against total RNA of rat, avian, porcine, ovine, and bovine tissues. Tissues included skeletal muscle, brain, stomach, heart, and liver (Figure 10). Data indicate detection of a 1.6 kb band corresponding to SKA transcript in total RNA from skeletal muscle samples was detected across all mammalian species. No cross-reactivity was noted across tissue types which express endogenous β and γ actin isoforms, or the CAA isoform, indicating

Figure 10 Species and tissue skeletal α-actin Northern blot

Depicted are the results of Northern blot analysis of 10 μg total RNA from select tissues and species hybridized against the mouse 105 bp 3' untranslated region probe. The results idicate that the 105 bp skeletal α-actin probe is specific for the skeletal α-actin isoform across several laboratory and livestock mammalian species. The species included mouse, rat, chicken, pig, cattle, and sheep. The specificity of the probe for the skeletal α-actin isoform is confirmed by the absence of binding in a other tissues expressing the non-muscle actin isoforms.

The tissues represented include skeletal muscle (SM), heart (CD), stomach (ST), liver (LR), and brain (BR). The samples representing the "stomach" in chicken and ruminant species were gizzard and abomasum, respectively.

Cross reactivity was identified at 1.8 kb in the rodent preparations however, the cross reactivity was not identified in non-rodent species.


SM
CD
ST
LR
BR
MOUSE
1.8 kb
1.6 kb
RAT
1.6 kb
CHICKEN
1.6 kb
PIG
1.6 kb
SHEEP
1.6 kb
CATTLE
1.6 kb

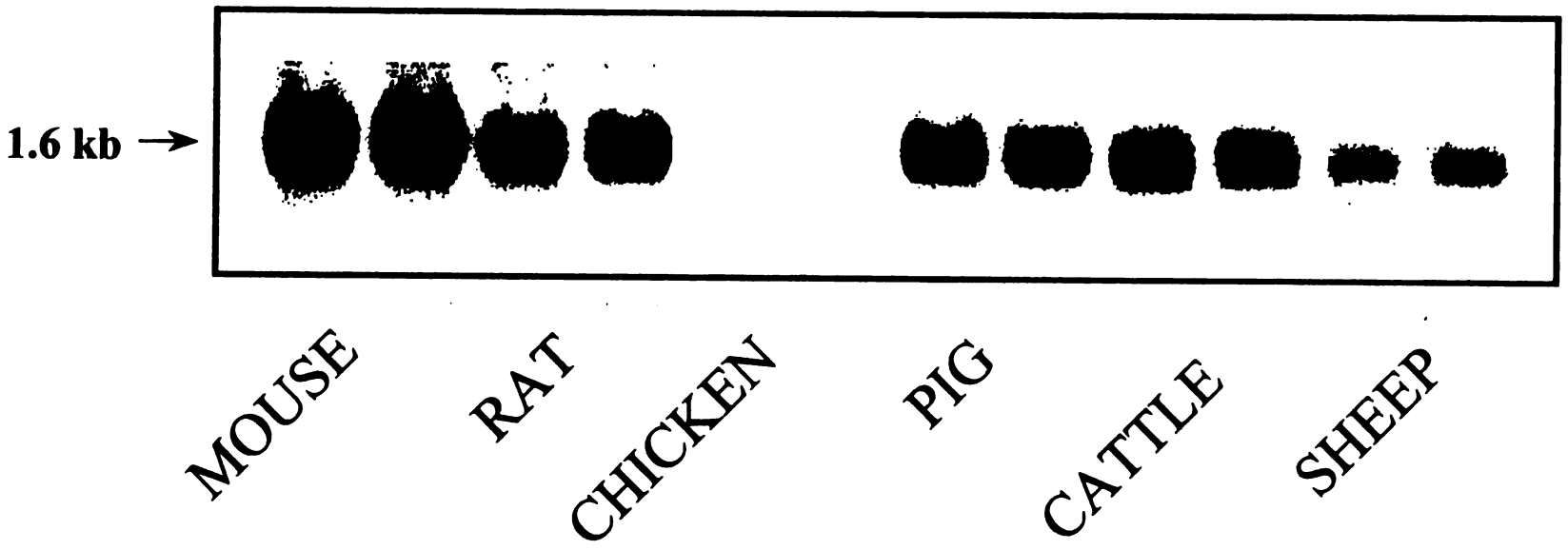
the specificity of the 105 bp probe for the SKA isoform (Figure 10). A transcript at a 1.8 kb migration point was also detected in rat skeletal muscle while not apparent in other species blots. The same 1.8 kb transcript appears in mouse skeletal muscle total RNA hybridizations and will be addressed further in the next section. No binding was observable in any chicken tissue total RNA samples. The fact that no binding was observed for any chicken tissue tested, especially skeletal muscle, is consistent with earlier studies (Shani, et al., 1981) where a cDNA probe homologous to a rat 3'UTR hybridized with mammalian species SKA but not with chicken. The results suggest that this 105 bp fragment is too divergent from the chicken SKA isoform and that the 105 bp fragment is specific for mammalian SKA (Figure 11).

Cross Reactivity at 1.8 kb

Slight cross-reactivity at approximately 1.8 kb in mouse skeletal muscle was observed (Figure 10). The cross-reactivity may be a pre-splice transcript or other homologous base pairing across unidentified RNA at the level of the 28S rRNA in rodent skeletal muscle. Minty et al., (1982) report that there approximately 20 actin-like genes in the mouse genome as determined by nucleic acid homology which have been reported across chromosomes. The band of cross-reactivity at a migration of ~1.8 kb may represent a rodent actin-like gene transcript which may share a degree of homology with the 105 bp probe created from the mouse SKA gene 3' UTR.

Figure 11 Species skeletal muscle skeletal α-actin Northern blot

The figure represents the Northern blot analysis of 10 μg total skeletal muscle RNA across several species. The specificity of the105 bp skeletal α-actin probe to the mammalian isoform of skeletal α-actin is demonstrated by the presence of binding at 1.6 kb in mammalian skeletal muscle total RNA samples and the complete absence of binding in the avian skeletal muscle sample.


1.6 kb
MOUSE
RAT
CHICKEN
PIG
CATTLE
SHEEP

It is unlikely that the band of cross-reactivity at 1.8 kb represents either the β or γ isoforms. These isoforms are typically detected at 2.1 kb, and hybridization with a transcript, at that particular molecular weight, was not apparent in the skeletal muscle sample or other tissues which would predominantly express those particular isoforms as well as in skeletal muscle.

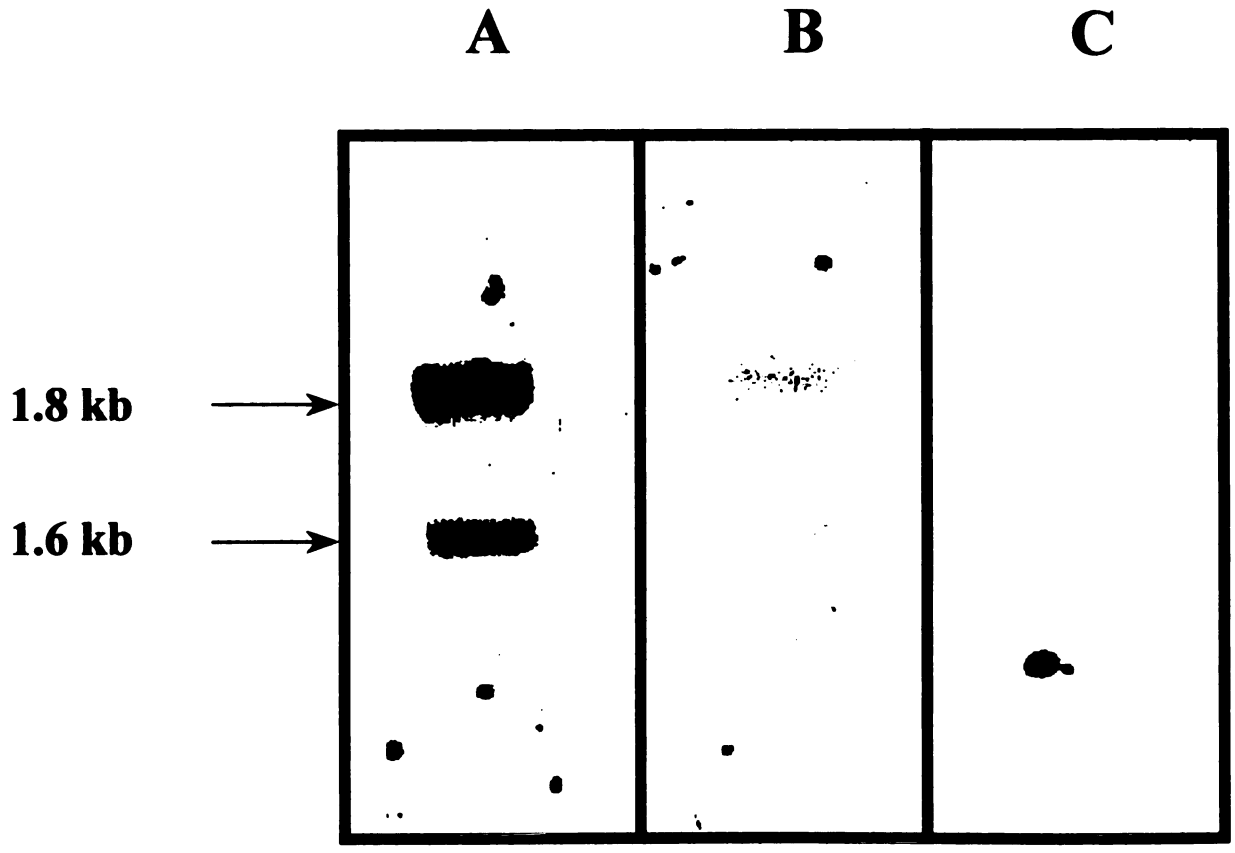
A competition experiment was performed with 1,000 fold excess of unlabeled 105 bp probe in a standard hybridization to examine the specificity of the binding at the 1.8 kb. The competition experiment suggested that the binding at 1.8 kb was specific as the intensity at 1.6 kb and 1.8 kb diminished in parallel to that of the 1.6 kb band (Figure 12). A BLAST homology search was performed on the 105 bp fragment and did not suggest any homologous base pairings with non-skeletal muscle α-actin isoforms. The same RNA blot was incubated without cDNA probe and exposed only to the anti-Dig Fab fragment to rule out the possibility that the antibody was leading to the detection of the cross-reactivity. Results with the antibody alone suggest that the anti-Dig Fab fragment is not influencing the cross-reactivity at 1.8 kb (Figure 12). The band of cross-reactivity appears to be present only in the rodent species and remains unidentified.

The PCR generated DNA probe homologous to a 105 bp region within the 3' UTR of the mouse SKA gene is SKA isoform specific across several mammalian livestock species. The results are consistent with the observation that the 3'UTR of the actins show great diversity across isoforms and species (Shani et al., 1981, Gunning et al., 1984) and that a

Figure 12 Probe competition experiment

The nature of cross-reactivity in total RNA samples of rodent skeletal muscle was addressed through a probe competition experiment.

Lanes represented, (A) 10 ug total RNA, mouse skeletal muscle, (B) same as A with 1,000 fold excess of unlabeled probe, (C) same as A, no probe in hybridization and detection procedure as indicated in methods section. Following detection, the bands at 1.6 and 1.8 kb diminish at the same intensity between (A) and (B) suggesting specific binding at 1.8 kb. Relative to (C), it appears that the Ab in the detection method was unlikely to cause the appearance of the band at 1.8 kb.


A
B
C
1.8 kb
1.6 kb

probe synthesized from that region may confer isoform specificity. The SKA-specific probe will be useful in examining SKA mRNA abundance as an index of myofibrillar gene expression.

CHAPTER 2

β-ADRENERGIC STIMULATION INCREASES SKELETAL α-ACTIN mRNA ABUNDANCE IN C_2C_{12} MYOTUBES

Abstract

We have previously reported that feeding a synthetic βAA to finishing pigs increases SKA mRNA abundance (Helferich et al., 1990). The objectives of this study were to characterize the SKA mRNA abundance during development and to determine the effect of βAA stimulation on abundance of SKA mRNA in C2C12 myotubes. Cells were seeded in 60 mm-diameter dishes, grown to confluence in DMEM containing 10% FBS. At confluence, media was changed to DMEM containing 2% FBS to induce differentiation. Cells were harvested at various stages of development, from proliferating myoblasts to differentiated myotubes. Myosin accumulation was measured by immunoblot analysis using an anti-sarcomeric myosin (SM) antibody (NA4). A PCR generated probe homologous to 105 bp of the 3' UTR of the mouse SKA gene was characterized and used to quantify SKA mRNA abundance. Skeletal α-actin mRNA abundance parallels SM accumulation in developing C2C12 cells. C2C12 myotubes were exposed to ISO at (10^{-9} M to 10^{-5} M), or FOR at (10^{-8} M to 10^{-4} M), for 48 hours, then harvested for RNA isolation. Isoproterenol treatment resulted in a dose dependent increase in SKA mRNA abundance with maximal expression at 10^{-5} M ($p<.05$). Maximal SKA mRNA abundance was observed at 10^{-4} M FOR ($p<.05$). Skeletal α-actin mRNA abundance of myotubes was increased by the addition of cAMP to culture media ($p<.05$). Isoproterenol-induced increases in SKA mRNA abundance were eliminated by

simultaneous addition of a β-receptor antagonist, propranolol (PRO), or the protein kinase A inhibitor, HA1004. These data indicate that expression of SKA mRNA parallels sarcomeric myosin accumulation in differentiating C2C12 myoblasts and that β-adrenergic stimulation increases SKA mRNA abundance via a protein kinase A-dependent pathway.

Introduction

The basic components of many of the major intracellular signaling pathways have been well established. Signaling pathways may be composed of a variety of proteins including specific receptors, GTP-binding proteins, second messenger generating enzymes, protein kinases, target functional proteins, and regulatory proteins (Nishizuka, 1992, Barnard, 1992). Interactions across signaling pathways are diverse and include potentiation, cooperation, synergism, antagonism, and co-transmission. The secondary or tertiary messengers that translate the signals into molecular responses at the level of gene expression are not understood and are presumably multifactorial (Iwaki et al., 1990). The growing list of such nuclear effector proteins include transcription factors and two tumor suppressor proteins (Meek et al., 1992). Several of these proteins are phosphorylated by the same protein kinases, suggesting that signals may coordinatedly target these key proteins (Meek et al., 1992). Although it is clear that βAR stimulation must generate intracellular mediators that reach the nucleus and consequently activate skeletal muscle specific gene expression, the precise signaling mechanisms that link the occupancy of the receptor with gene induction remain unresolved (Iwaki et al., 1990). Previous research (Bishopric et al., 1992) indicated a direct relationship between specific transcription factor activation (c-fos, c-jun) and signal transduction pathway cross-talk in stimulating expression of the SKA gene which was transiently transfected into cardiac myocytes.

The same regulatory elements present in the promoter of the transfected SKA reported by Bishopric et al. (1992) are present in the endogenous SKA. It seems unlikely that the SKA promoter would function any differently in vivo. Figure 1 depicts possible interaction across mutiple signal transduction pathways and the potential involvement in the regulation of SKA mRNA abundance in skeletal muscle.

It has been reported that feeding of βAA to livestock leads to increased mRNA abundance for several myofibrillar proteins, including SKA, myosin light chains 1 and 3 and the protease inhibitor, calpastatin (Helferich et al., 1990, Smith et al., 1989, Koohmaraie et al., 1991). These studies did not explain the mechanism regulating the increased mRNA abundance in βAA treated animals. Considering total RNA concentration is an indirect measure of protein synthetic capacity and that mRNA repesents the precursor to translation, an increase in the relative abundance of a specific mRNA indicate an increase in protein synthetic capacity (Hakkarainen, 1975, Waterlow et al., 1978). Shani et al., (1981) demonstrated that transcription of new mRNA is directly responsible for the onset of protein synthesis in vitro.

Based on the previously mentioned studies, the objectives of these experiments were to (1) demonstrate that βAA's increase SKA mRNA abundance and (2) define the pathway of βAA increased SKA mRNA abundance in C_2C_{12} myotubes.

Materials and Methods

Cells

C_2C_{12} muscle cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Approximately 50,000 cells/dish were seeded into 60 mm diameter dishes in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, Inc., Grand Island, NY) containing 10% fetal bovine serum (FBS; Sigma Chemical Co., St. Louis, MO.). Cells were grown to confluence in DMEM containing 10% FBS in a humidified atmosphere of 5% CO_2 and 95% air at 37° C. Upon confluence, growth media was replaced with differentiation media (DMEM, 2% FBS) for approximately 6 days to stimulate myotube formation. Medium was replaced every 48 hours unless otherwise indicated during the experiments.

Sample Preparation for Immunoblot Analysis

Myotube cultures were rinsed three times in phosphate buffered saline, and harvested using a low salt NFEP homogenization buffer (.05 M NaF, .01 M EDTA, .075 M NaCl, .015 M Na_2HPO_4, pH 7.0) and sonicated using Branson Sonic Power Co., Sonifier-Cell disrupter 350 at setting #4 for ~5 seconds. Aliquots of each sample were saved for protein and DNA quantification and for SDS-PAGE. Protein was determined by the method of Bradford (Bradford, 1976) using commercial reagents (Bio-Rad, Hercules,

CA). DNA was determined fluorometrically using the Hoescht 33258 reagent (West, 1985). Sample aliquots for SDS-PAGE were prepared by mixing equal portions of the aliquot and hot 2x treatment buffer (125 mM Tris, 4% SDS, 20% glycerol, 10% mercaptoethanol, pH 6.8). Samples not used immediately were stored at -80° C and heated for 5 minutes at 95°C before loading onto gels.

SDS-PAGE and Western Blotting

Samples were loaded on an equal protein basis on an 8% polyacrylamide (37.5:1, acrylamid:N,N-methylenbisacrylamid solution) (Bio-Rad Laboratories, Hercules, CA) separating gel with a 4% polyacrylamide stacking gel and fractionated at 165 volts for approximately 1 hour. Proteins were transferred to Immobilon P membrane (Millipore, Inc., Bedford, MA), dried, and stored until blotting. Initially, membranes were blocked for 1 hour in blocking buffer (1% BSA, .05% Tween 20 in Tris-buffered saline) followed by incubation with the anti-sarcomeric myosin antibody NA-4 (kindly provided by Dr. E. Bandman, U.C. Davis). Following primary Ab incubation, blots were further incubated with an anti-mouse secondary Ab conjugated to either alkaline phosphatase (AP) or horseradish peroxidase (HRP) (1:20,000) (Sigma Immunochemicals, St. Louis, MO). Membranes were washed (3 x 5 minutes) in blocking buffer following each Ab incubation. Ab binding was visualized by exposure of membranes to colorimetric AP reagent (BCIP/NBT; Bio-Rad, Hercules, CA) or chemilluminescent HRP substrate (Super-Signal Chemilluminescent Substrate for Western Blotting; Pierce, Rockford, IL).

Plasmids

Skeletal α-actin Plasmids

Inconsistencies in the pMACT-α plasmid map required verification of the pMACT-α sequence. A 931 bp BamHI fragment corresponding to bp 3071-4002 of the mouse SKA gene in pMACT-α (Minty and Kedes, 1987) was subcloned into the plasmid pcDNA3 (Stratagene, Inc., La Jolla, CA) for sequence verification. Following the sequence verification of the 931 bp fragment, a 437 bp Pst-I/BamHI fragment corresponding to bp 3565-4002 of the mouse SKA gene was subcloned into pBluescript II SK+/- (Stratagene, Inc., La Jolla, CA). The intentions were to allow for maximal replication of the 451 bp fragment to be collected by plasmid preparation for use as a PCR template. Bacteria were transformed and screened on X-gal plates (Maniatis et al., 1982). Plasmid preps were performed on selected colonies. A set of appropriate primers was chosen for PCR and the 105 bp fragment amplified through a "touch-down" procedure. After the initial PCR, the non-labeled 105 bp fragment was used as template for amplification by PCR (see Appendix A).

18S rRNA Plasmid

The plasmid pN29III (Oberbaumer, I., 1992), which contained as an insert the mouse 18S rRNA gene, was obtained from the American Type Culture Collection (ATCC, Rockville, MD). The plasmid was amplified in bacterial culture and isolated via plasmid preparation. A Bam HI/ Sph I digest of the plasmid yielded a 750 bp fragment which was utilized as a template for PCR. A set of appropriate primers was chosen for PCR and the 546 bp fragment was amplified through a "touch-down" procedure (Maniatis et al., 1982,

see Appendix D). After the initial PCR, the non-labeled 546 bp fragment was purified on an 1.2% agarose gel and used as template for amplification by PCR.

Primers

Skeletal α-actin

Primers were chosen according to accepted protocol (Maniatis et al, 1982). The primers were prepared at the Michigan State University Macromolecular Structure Facility. The oligonucleotides:

WGB1 (5' TTG GAG CAA AAC AGA ATG GCT G 3') and

WGB2 (5'ATA GAT TGA CTC GTT TTA CCT CA 3')

were chosen as primers for PCR and represented sequences complementary to bp 3674-3696 and 3591-3612 bp of the mouse skeletal α-actin gene, respectively.

18s rRNA

Primers were chosen according to accepted protocol (Maniatis et al., 1982). The primers were prepared at the Michigan State University Macromolecular Structure Facility. The oligonucleotides:

MED1 (5' AGC ATA TGC TTG TCT CAA AG 3') and

MED2 (5' GGA CTC ATT CCA ATT ACA GG 3')

were chosen as primers for PCR and represented sequences complementary to bp 1211-1230 and 1738-1757 bp of the mouse 18S rRNA gene, respectively .

Polymerase Chain Reaction
Skeletal α-actin

A digoxygenin labeled probe was created using the Boehringer Mannheim Biochemicals-Digoxygenin PCR Kit (BMB, Indianapolis, IN). The Bam HI/Pst-I 451 bp fragment was the initial template and the oligonucleotide primers WGB 1/2 were used in a "touch-down" procedure. The amplified fragment was size verified using a 100 bp DNA ladder (Gibco, Grand Island, NY) at 105 bp and purified from an agarose gel using the Prep-A-Gene kit from Bio-Rad. The "touch-down" procedure allows for efficient amplification of templates which have calculated higher annealing temperatures (>50) (see Appendix A).

18S rRNA

A digoxygenin labeled probe was created using the Boehringer Mannheim Biochemicals-Digoxygenin PCR Kit (BMB, Indianapolis, IN) using the Bam HI/Sph I 750 bp fragment as the initial template and the oligonucleotide primers MED 1/2 in a "touch-down" procedure. The amplified fragment was size verified using a 100 bp DNA ladder (Gibco, Grand Island, NY) at 546 bp and purified from an agarose gel using the Prep-A-Gene kit from Bio-Rad. The "touch-down" procedure allows for efficient amplification of templates which have calculated higher annealing temperatures (>50) (see Appendix D).

Sequencing
Skeletal α-actin Fragments

A 931 bp Bam HI fragment subcloned in the initial plasmid clone pS3'α of pcDNA3 was sequenced at the Michigan State University Plant Research Laboratory using the

Dyedeoxy procedure from the T7 dye primer or Sp6 dye primer, a system designed by Applied Biosystems Inc. The Dyedeoxy method of sequencing is similar to the Sanger dideoxy method which represents a controlled interruption of enzymatic replication. The Dyedeoxy chromatogram is pictured in Appendix B.

The 105 bp fragment obtained from the PCR reaction using the Bam HI/Pst-I 451 bp fragment of the pBluescript plasmid clone and WGB1/2 primers was sequenced using the Sequenase protocol (United States Biochemical (USB), Cleveland, OH). The sequence of the 105 bp fragment was verified by comparison to the published sequence of mouse SKA on GenBank (Name: MUSACASA, Accession: M12347) to be homologous to 3591 bp through 3696 bp of the 3' UTR of the mouse SKA gene.

18S rRNA 546 bp Fragment

The 546 bp fragment representing the 18S rRNA was sequenced using a dye-deoxy procedure with the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Norwalk, CT). The chromatogram is presented in Appendix E. The 546 bp fragment is homologous to 1211 bp to 1757 bp of the rat 18S rRNA.

RNA Isolation

Total RNA was isolated from C2C12 myotube cultures in 60 mm diameter dishes using 1 ml Trizol reagent (GIBCO BRL. Co., Grand Island, NY). Myotubes were scraped from the 60 mm dishes with a rubber policeman then allowed to sit at room temperature for

fifteen minutes to allow for the complete dissociation of nucleoprotein complexes before an initial pre-spin at 12,000 x g for 5 minutes at 4° C. The supernatant was then extracted against chloroform (.2 ml chloroform per 1 ml Trizol reagent), and phase separation achieved by centrifugation at 12,000 x g for 15 minutes at 4° C. The RNA was precipitated from the extract with isopropanol (.5 ml per 1 ml Trizol reagent) stored on ice for 10 minutes, then centrifuged at 12,000 x g for 20 minutes at 4° C. The RNA pellet was then washed by vortexing with 75% ethanol (1 ml per 1 ml Trizol reagent) and centrifuged at 7,500 x g for 5 minutes at 4° C. The RNA pellet was resuspended in sterile milli-Q diethylpyrocarbonate (DEPC) treated RNase free water and stored at -80° C until analysis. RNA solutions were scanned from 220 nm - 320 nm, the A260/A280 ratio was determined, and RNA concentration calculated from the A260. Typical total RNA yield from a 60 mm dish containing mature myotubes was approximately 100 μg.

Northern Blot Analysis

Ten micrograms total RNA extracted from myotube samples was electrophoretically separated on a denaturing 1.2% agarose, 2.2 M formaldehyde gel at ~60 V for 3 hours. A control RNA, isolated from mouse hind-limb muscle, was used in all studies to aid in the normalization of hybridization data across studies. RNA was transferred to positively charged nylon membranes (Boehringer Mannheim Biochemicals, Indianapolis, IN) overnight with a 10x SSC solution. Following Northern transfer, the membranes were allowed to dry slightly and were then UV crosslinked using a Spectroline transilluminator (model # 302) for 3 minutes before prehybridization in a standard

hybridization buffer (5X SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, and 1% blocking reagent) for 8 hours. Blots were hybridized with a digoxygenin labeled 105 bp PCR generated SKA probe at 42° C for 8 hours. After hybridization, membranes were washed (2 x 5 minutes) in a 2x SSC/ .1% SDS solution at 42° and then (2 x 15 minutes) in a .1x SSC/ .1% SDS solution at 42° C. Membranes were blocked for 30 minutes and then exposed to anti-Dig Fab fragments (1:15,000) (Boehringer Mannheim Biochemicals, Indianapolis, IN) for 30 minutes. Membranes were washed (2 x 15 minutes) in a .3% Tween-20 Maleic acid buffer (100 mM maleic acid, 150 mM NaCl; pH 7.5), rinsed in a Tris buffer (100 mM Tris-HCl, 100 mM NaCl; pH 9.5) for one minute and exposed to the chemilluminescent AP detection substrate, CDP-*Star* (Boehringer Mannheim Biochemicals, Indianapolis, IN), for five minutes. Membranes were then sealed in plastic bags and exposed to x-ray film. Hybridization of RNA to the 105 bp SKA probe was quantified by videodensitometry. Size was verified at 1.6 kb using an RNA marker (Promega Co., Madison, WI). Following the SKA hybridization, the blots were stripped by a 5-minute wash in DEPC water followed by a boiling DEPC water/ 0.1% SDS (w/v) wash for 10 minutes. The blots were then placed in the prehybridization buffer for 8 hours then incubated with an 18S rRNA probe. Detection of the 18S rRNA was performed as previously mentioned for detection of SKA mRNA. The level of expression of the SKA mRNA was calculated as the ratio of the intensity of the SKA band to the intensity of the 18S rRNA band. This presents the level of specific mRNA per unit rRNA and minimizes any variation in loading of the gel. The ratios are expressed as percentages of the mean value for control tissue cultures on each

blot to enable direct comparison between blots. RNA data were expressed per 18S rRNA abundance and relative to 2% FBS control treatment. The 18S rRNA band was utilized as a constituitively expressed, non-fluctuating marker. The rRNA represents approximately 85% of all total RNA in the cell and codes for the proteins involved in the ribosomal translational machinery.

Muscle Creatine Kinase Activity

Cells at each time point of the developmental time course, from rapidly dividing myoblasts to mature myotubes, were washed 3 times in PBS, overlaid with 200 ul .05 M glycylglycine buffer, pH 6.75, and stored at -80° C until assay. The cells were scraped from plates using a rubber policeman, sonicated, and a 10 ul sample assayed for creatine kinase activity using the Sigma (47-UV) kit and manufacturer's instructions (Sigma Chemical Co., St. Louis, MO.) (Szasz et al., 1976). DNA was quantified spectrophotometrically using the Hoescht 33258 reagent as described by West et al. (1985) using calf thymus DNA as the standard. Creatine kinase activity was expressed per unit DNA.

Developmental Time Course

RNA was isolated from C_2C_{12} cells at various stages of development, from rapidly dividing myoblasts to mature myotubes. Ten micrograms total RNA were loaded per lane and electrophoretically separated as described previously. The RNA was transferred to a positively charged nylon membrane and subjected to Northern blot analysis with the

SKA and 18S rRNA probes. Bands were quantified using videodensitometry using the Bio-Rad MultiAnalyst System (Bio-Rad, Hercules, CA).

Skeletal α-actin mRNA Abundance in Response to Increasing Duration of Isoproterenol Stimulation

Maximal response of SKA mRNA abundance as a result of βAA stimulation was determined over a series of time points post differentiation. Cells were maintained as previously described. Fresh treatment media (10^{-5} M ISO in DMEM/2% FBS) were applied at 24 hour intervals beginning at 6 days after addtion of fusion media to cells. Time points included a 72, 48, 24, 8, 4, and 1 hour. Treatments 8, 4, and 1 hour were created in present media and returned to plates for the assigned time interval to minimize the response of the cells to fresh media and also keep treatments consistent across time points.

β-adrenergic Pathway Component Stimulation

Stimulation of various components of the βAA stimulatory pathway may indicate the role of each component in eliciting the βAA stimulated increase in SKA mRNA abundance. Key points in the βAA pathway include the G_s protein, AC and PKA, stimulated by CTOX (Sigma Chemical Co., St. Louis, MO.), FOR (Sigma Chemical Co., St. Louis, MO.), and cAMP (Sigma Chemical Co., St. Louis, MO.), respectively. The fact that several protein kinases may converge on the same transcriptional activator suggests that more than a single pathway may be involved in βAA stimulation of SKA transcription.

Involvement of PKA and PKC pathways was addressed by including PE (Sigma Chemical Co., St. Louis, MO.), a potent activator of PKC. Other pathway component modulators included the potent inhibitors of PKA and PKC; HA1004 (ICN, Costa Mesa, CA), and staurosporine (Sigma Chemical Co., St. Louis, MO.) respectively, and H7 (Sigma Chemical Co., St. Louis, MO.), a potent inhibitor of both PKA and PKC. Inhibitors were preincubated for one hour before the addition of specified agonist.

Statistical Analysis

Data were analyzed through analysis of variance by using the program of SAS (SAS, 1996). Differences among means were tested for significance ($P<.05$) using the method of Tukey or comparison across treatments by contrast.

Results and Discussion

Skeletal α-actin mRNA/Sarcomeric Myosin /Muscle Creatine Kinase Activtiy Developmental Time Course

The SKA mRNA band at 1.6 kb progressively increased in intensity during differentiation of C2C12 muscle cells. Initial appearance of the 1.6 kb band was approximately 48 hours post confluence and paralleled SM protein accumulation (Figure 13). The developmental pattern of expression of SKA mRNA and the SM is consistent with the established expression of muscle-specific expression of key contractile proteins. Caravatti et al., (1982) determined that the corresponding mRNAs coding for myosin heavy chain and for α-actin are detectable immediately before the initiation of myofibrillar protein synthesis. Devlin and Emerson (1978) reported that myofibrillar proteins accumulate at the same time, have similar synthetic rates, and reach steady state levels at the same time. The results presented by Caravatti et al. (1982) demonstrated a close temporal correlation between muscle mRNA accumulation and protein synthesis during myogenesis. Muscle creatine kinase activity, which was measured over a similar developmental time course, followed a similar pattern to that of the developmentally expressed SKA mRNA and SM, reaching peak activity at differentiation (Figure 14). An RNA sample was obtained at each time point parallel to the sample obtained for MCK and exhibited expression consistent with the pattern observed for the previous SKA

Figure 13 Skeletal α-actin mRNA and sarcomeric myosin developmental time course in C2C12 muscle cells

Characterization of the C2C12 cell line was accomplished partially through an examination of skeletal α-actin mRNA abundance and sarcomeric myosin protein across a developmental time course. Total RNA and cell extracts were prepared from proliferating, confluent, and differentiating C2C12. Columns represented include (R) rapidly dividing myoblasts, (C) confluent myoblasts, (1D) one day post-confluence, (2D) two days post-confluence, (3D) three days post-confluence, (4D) four days post-confluence, (5D) five days post-confluence, and (6D) six days post-confluence.

Total RNA and cell extracts were prepared as described. Northern and immunoblots were quantified using videodensitometry and results expressed in parallel to attest the similar pattern of expression of skeletal α-actin mRNA abundance and sarcomeric myosin protein expression across development.

The graph depicts the parallel developmental expression of skeletal α-actin mRNA and sarcomeric myosin protein accumulation in C2C12 cells indicating that these cells exhibit the myogenic phenotype.

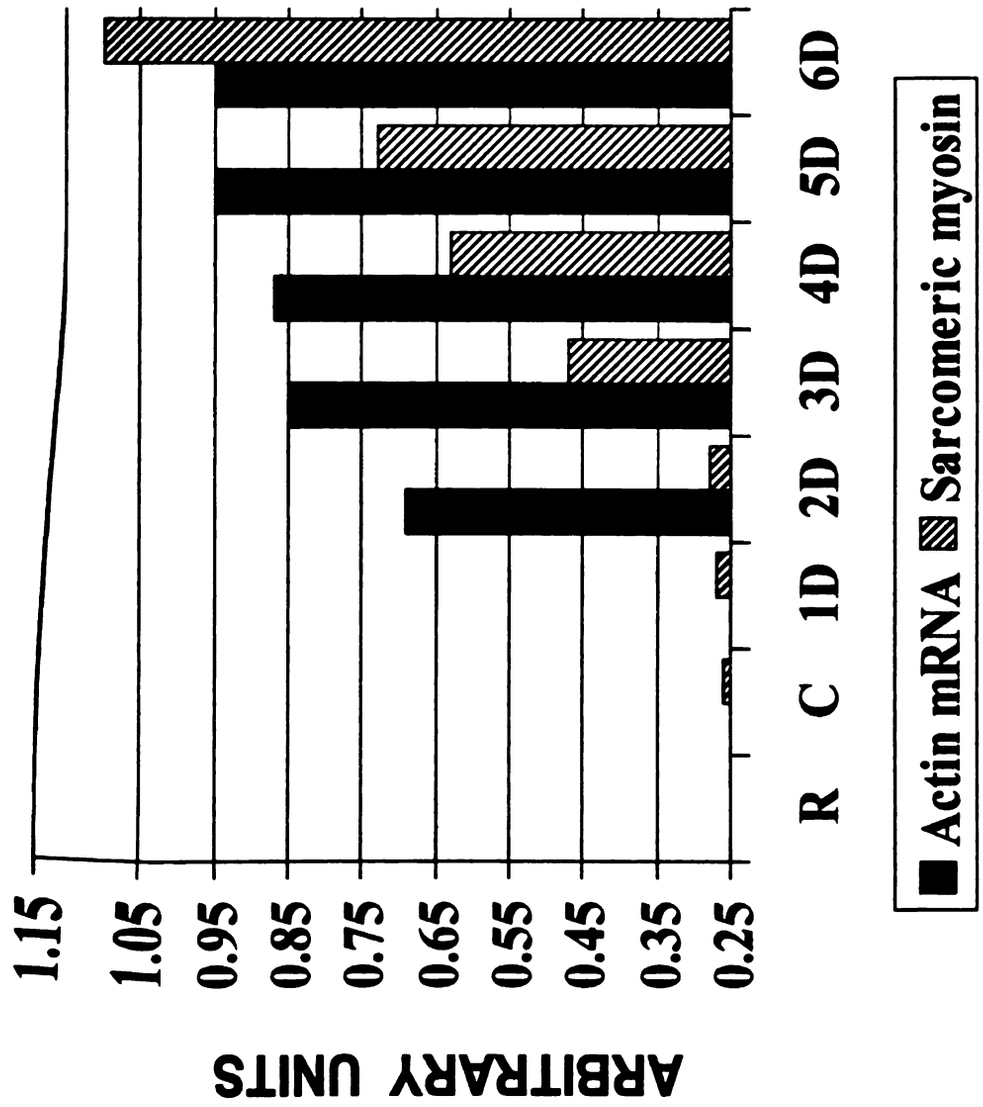

ARBITRARY UNITS
1.15
1.05
0.95
0.85
0.75
0.65
0.55
0.45
0.35
0.25
R C 1D 2D 3D 4D 5D 6D
Actin mRNA
Sarcomeric myosin

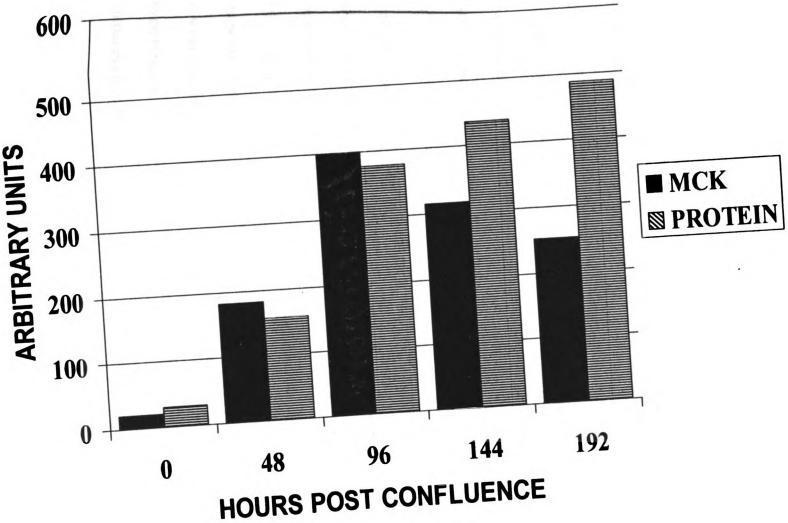
Figure 14 Muscle creatine kinase enzyme activity and protein accumulation over a developmental time course in C2C12 muscle cells

Characterization of the C2C12 cell line was accomplished partially through an examination of muscle creatine kinase enzyme activity across a developmental time course. Cell extracts were prepared from confluent, proliferating, and differentiating C2C12 cells. Columns represented include (0) confluent myoblasts, (48) 48 hours post confluence, (96) 96 hours post-confluence, (144) 144 hours post-confluence, and (192) 192 hours post-confluence.

Cell extracts were prepared as described. Muscle creatine kinase acitivity was expressed on a DNA basis. Protein was determined according to the method of Bradford (1976).

The graph depicts the developmental expression of muscle creatine kinase enzyme activity and protein accumulation through development in post-confluent C2C12 cells. The data agree with the documeted developmental expression of muscle creatine kinase activity in skeletal muscle.

The data further validate the capacity of the C2C12 cell line to exhibit the myogenic phenotype and as a model to study muscle specific biochemistry in vitro.


600
500
400
300
200
100
0
ARBITRARY UNITS
0
48
96
144
192
HOURS POST CONFLUENCE
MCK
PROTEIN

mRNA and SM data. The developmental data represents the characteristic expression of key components in skeletal muscle differentiation and suggests that the C2C12 system is an appropriate in vitro system in which to study muscle-specific gene/protein expression.

Isoproterenol Dose Response

A βAA dose response study was undertaken utilizing the non-selective βAR agonist ISO. Myotubes were exposed to ISO at 10^{-9} M to 10^{-5} M for 48 hours, then harvested for RNA isolation. Isoproterenol treatment resulted in a dose-dependent increase in SKA mRNA abundance with maximal expression at either 10^{-5} M (Figure 15). Surprisingly, SKA mRNA abundance was lower ($p<.05$) than the 2% FBS control at low concentrations of ISO (10^{-9}-10^{-6} M). This response appears to be due to a decrease in SKA mRNA and not a result of expressing the data per 18S rRNA. Separate SKA mRNA and 18S rRNA values are plotted tandemly in Figure 16. No differences in 18s rRNA were detected. Presently, no explanation is available for the decrease in SKA mRNA abundance at lower ISO concentrations. Further, no change in total protein was observed across the dose-response study (Figure 17). Although contrary to βAA-stimulated protein accumulation in vivo, protein accretion in vitro may be limited by restrictions relative to other hypertrophic stimuli, such as stretch, nervous stimulation, and contact inhibition in cultured myotubes.

Figure 15 Isoproterenol dose response on skeletal α-actin mRNA abundance in C2C12 myotubes

A β–adrenergic agonist dose response study was undertaken to determine the optimal concentration of the non-selective β-adrenergic agonist, isoproterenol, to stimulate maximal skeletal α-actin mRNA abundance in C2C12 myotubes (n=4). Cells were grown as described. C2C12 myotubes were stimulated for 48 hours with increasing concentrations of isoproterenol (10^{-9} M -10^{-5} M). Fresh media was applied every 24 hours. At 48 hours, cells were harvested for isolation of total RNA.

Ten micrograms total RNA from C2C12 myotubes were run on a 1.2% agarose denaturing gel, transferred to nylon membrane and hybridized against a 105 bp skeletal α-actin probe. The same blots were stripped and rehybridized with an 18S rRNA probe. Following non-radioactive detection methods, the blots were analyzed using videodensitometry. The skeletal α-actin data are expressed per unit of 18s rRNA.

Data indicate maximal expression of skeletal α-actin mRNA abundance at 10^{-5} M isoproterenol relative to 2% FBS control ($P<.05$). Surprisingly and presently unexplainable, the lower concentrations of isoproterenol appeared to depress skeletal α-actin mRNA abundance relative to the 2% FBS control.

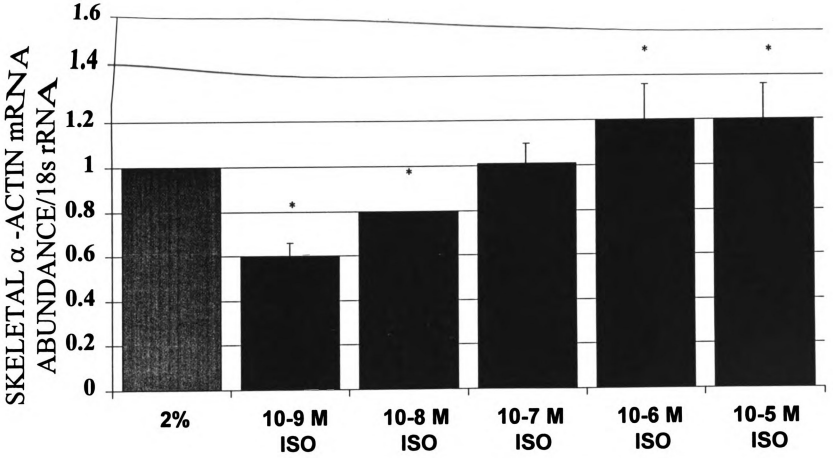

SKELETAL α -ACTIN mRNA ABUNDANCE/18s rRNA
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
2%
10-9 M ISO
10-8 M ISO
10-7 M ISO
10-6 M ISO
10-5 M ISO
*
* Means significantly different from 2% FBS control, (p<.05).

Figure 16 Isoproterenol dose response on skeletal α-actin mRNA and 18S rRNA abundance in C2C12 myotubes

A β-adrenergic agonist dose response study was undertaken to determine the optimal concentration of the non-selective beta-adrenergic agonist, isoproterenol, to stimulate maximal skeletal α-actin mRNA abundance in C2C12 myotubes (n=4). Cells were grown as described. C2C12 myotubes were stimulated for 48 hours with increasing concentrations of isoproterenol (10^{-9} M -10^{-5} M). Fresh media was applied every 24 hours. At 48 hours, cells were harvested for isolation of total RNA.

Ten micrograms total RNA from C2C12 myotubes were run on a 1.2% agarose denaturing gel, transferred to nylon membrane and hybridized against a 105 bp skeletal α-actin probe. The same blots were stripped and rehybridized with an 18S rRNA probe. Following non-radioactive detection methods, the blots were analyzed using videodensitometry.

The data presented represent the skeletal α-actin mRNA abundance and 18S rRNA desitometric values before being expressed as a ratio. The tandem plot illustrates the fact that the skeletal α-actin mRNA abundance is less at low concentrations of isoproterenol and not the result of data expression per unit 18S rRNA.

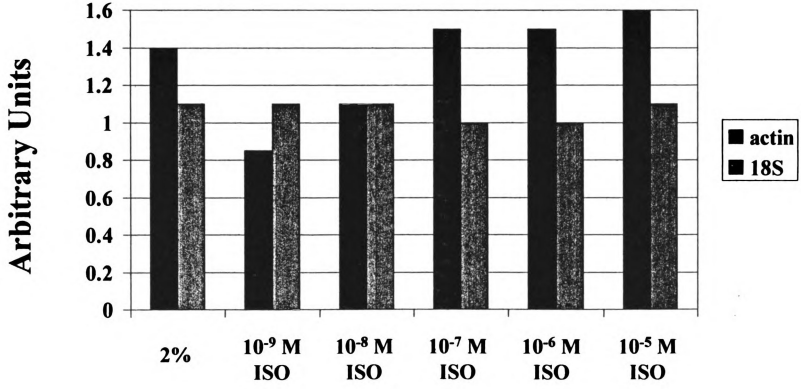
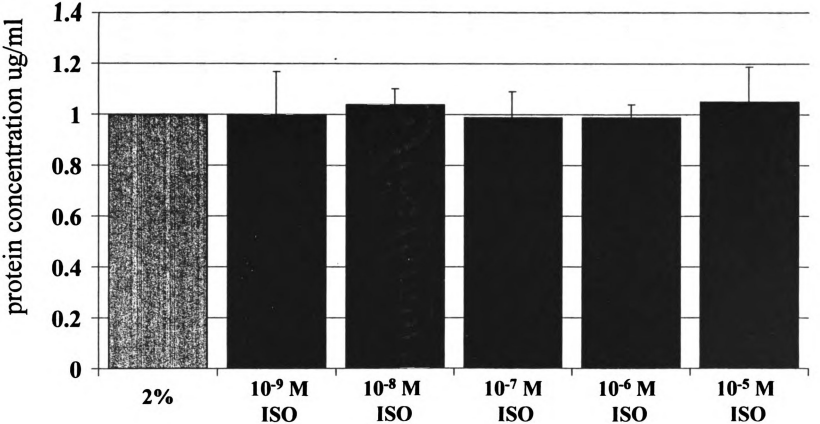

Arbitrary Units
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
2%
10-9 M ISO
10-8 M ISO
10-7 M ISO
10-6 M ISO
10-5 M ISO
actin
18S

Figure 17 Isoproterenol dose response on total protein abundance in C2C12 myotubes

A β-adrenergic agonist dose response study was undertaken to determine the optimal concentration of the non-selective β-adrenergic agonist, isoproterenol, to stimulate maximal skeletal α-actin mRNA abundance in C2C12 myotubes (n=4). Cells were grown as described. C2C12 myotubes were stimulated for 48 hours with increasing concentrations of isoproterenol (10^{-9} M -10^{-5} M). Fresh media was applied every 24 hours. At 48 hours, cells were harvested for determination of protein concentration.

Cells were washed 3 times with 1 ml PBS, scraped from plates using 200 µl NFEP buffer, and sonicated briefly. Protein concentration was determined by the method of Bradford (1976) in a microtiter plate and read spectrophotometrically at 550 nm. Protein concentrations were calculated relative to a bovine serum albumen standard curve.

Data indicate no significant difference across the isoproterenol concentrations relative to the 2% FBS control.


protein concentration ug/ml
1.4
1.2
1
0.8
0.6
0.4
0.2
0
2%
10-9 M ISO
10-8 M ISO
10-7 M ISO
10-6 M ISO
10-5 M ISO

Forskolin Dose Response

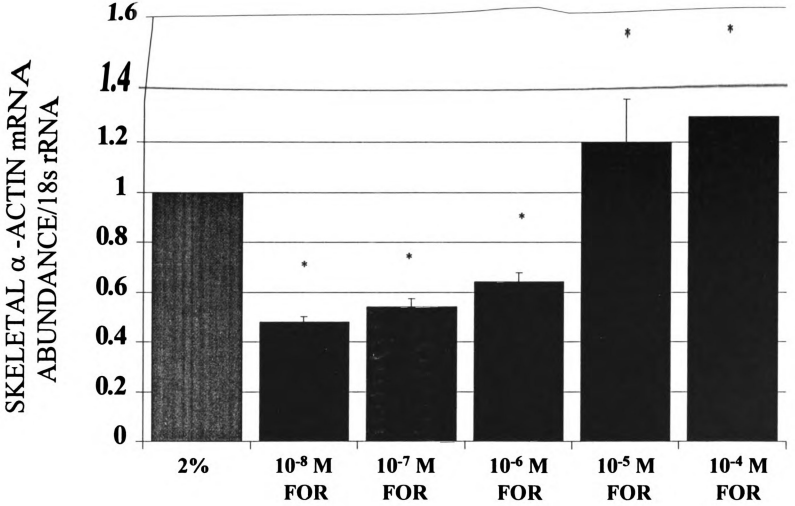
Forskolin is a potent stimulator of AC which increases levels of intracellular cAMP. Stimulation of AC by FOR should result in an increased level of SKA mRNA abundance as observed with βAA treatment, since AC is a key component in the βAA signaling pathway. Myotubes were exposed to FOR at 10^{-8} M to 10^{-4} M for 48 hours, then harvested for RNA isolation. FOR treatment resulted in a dose-dependent increase in SKA mRNA abundance with maximal expression at 10^{-4} M (Figure 18). As observed in the ISO dose response, SKA mRNA abundance was lower ($p<.05$) than 2% FBS control SKA mRNA abundance at low FOR concentrations (10^{-8}-10^{-5} M). In the case of FOR: DMSO was used as a carrier, however, the effect of the carrier on decreased SKA mRNA abundance must be considered irrelevant considering higher concentrations of FOR contained greater amounts of DMSO and the response was incremental with increasing FOR concentration. DMSO volume never exceeded 8 ul in the 2 ml media/dish. The SKA mRNA and 18S rRNA abundance values are tandemly plotted in Figure 19. No differences in 18S rRNA across treatments were observed. The data suggest that, as in the ISO dose response, SKA mRNA abundance decreases at the lower concentrations of FOR. No explanation is available at this time to describe the decrease in SKA mRNA abundance at the lower concentrations of either ISO or FOR. Consistent with findings in the ISO dose response experiment, no significant increase in total protein was observed in response to FOR (Figure 20).

Figure 18 Forskolin dose response on skeletal α-actin mRNA abundance in C2C12 myotubes

β-adrenergic stimulation by isoproterenol stimulates increased skeletal α-actin mRNA abundance in C2C12 myotubes. A β-adrenergic pathway component modulator dose response study was undertaken to determine the optimal concentration of the potent activator of adenylate cyclase, forskolin, to stimulate maximal skeletal α-actin mRNA abundance in C2C12 myotubes (n=4). Cells were grown as described. C2C12 myotubes were stimulated for 48 hours with increasing concentrations of forskolin (10^{-8} M -10^{-4} M). Fresh media was applied every 24 hours. At 48 hours, cells were harvested for isolation of total RNA.

Ten micrograms total RNA from C2C12 myotubes were run on a 1.2% agarose denaturing gel, transferred to nylon membrane and hybridized against a 105 bp skeletal α-actin probe. The same blots were stripped and rehybridized with an 18S rRNA probe. Following non-radioactive detection methods, the blots were analyzed using videodensitometry. The skeletal α-actin data are expressed per unit of 18s rRNA.

Data indicate maximal expression of skeletal α-actin mRNA abundance at 10^{-4} M forskolin relative to 2% FBS control ($P<.05$). Surprisingly and presently unexplainable, the lower concentrations of forskolin appeared to significantly depress skeletal α-actin mRNA abundance relative to the 2% FBS control ($p<.05$).



* Means significantly different from 2% FBS control, ($p<.05$).

Figure 19 Forskolin dose response on skeletal α-actin mRNA and 18S rRNA abundance in C2C12 myotubes

A dose response study was undertaken to determine the effect of the potent activator of adenylate cyclase, forskolin, on skeletal α-actin mRNA abundance in C2C12 myotubes (n=4). Cells were grown as described. C2C12 myotubes were stimulated for 48 hours with increasing concentrations of forskolin (10^{-8} M -10^{-4} M). Fresh media were applied every 24 hours. At 48 hours, cells were harvested for isolation of total RNA.

Ten micrograms total RNA from C2C12 myotubes were run on a 1.2% agarose denaturing gel, transferred to nylon membrane and hybridized against a 105 bp skeletal α-actin probe. The same blots were stripped and rehybridized with an 18S rRNA probe. Following non-radioactive detection methods, the blots were analyzed using videodensitometry.

The data presented represent the skeletal α-actin mRNA abundance and 18S rRNA desitometric values before expressed as a ratio. Skeletal α-actin mRNA abundance is significant at 10^{-4} M forskolin, expressed alone or per unit 18S rRNA. The tandem plot illustrates the fact that the skeletal α-actin mRNA abundance is significantly less ($p<.05$) at low concentrations of forskolin and not the result of data expression per unit 18S rRNA.

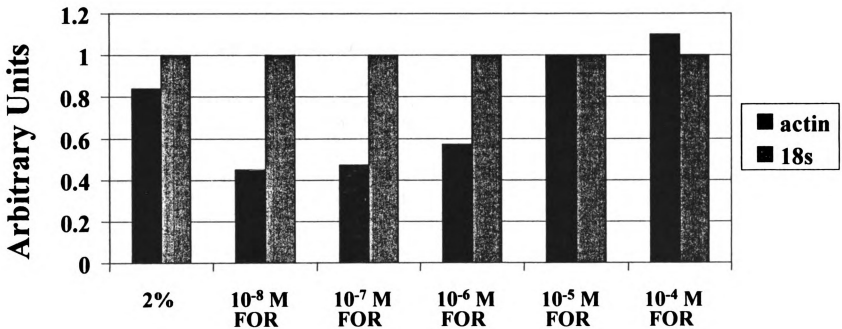
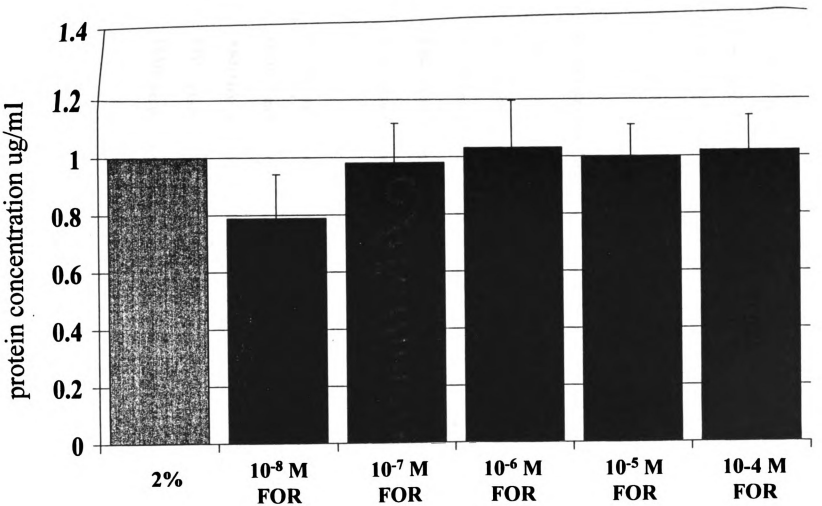

Arbitrary Units
1.2
1
0.8
0.6
0.4
0.2
0
2%
10-8 M FOR
10-7 M FOR
10-6 M FOR
10-5 M FOR
10-4 M FOR
actin
18s

Figure 20 Forskolin dose response on total protein abundance in C2C12 myotubes

A dose response study was undertaken to determine the effect of the potent activator of adenylate cyclase, forskolin, on skeletal α-actin mRNA abundance in C2C12 myotubes (n=4). Cells were grown as described. C2C12 myotubes were stimulated for 48 hours with increasing concentrations of forskolin (10^{-8} M -10^{-4} M). Fresh media were applied every 24 hours. At 48 hours, cells were harvested for determination of protein concentration.

Cells were washed 3 times with 1 ml PBS, scraped from plates using 200 µl NFEP buffer, and sonicated briefly. Protein concentration was determined by the method of Bradford (1976) in a microtiter plate and read spectrophotometrically at 550 nm. Protein concentrations were calculated relative to a bovine serum albumen standard curve.

Data indicate no significant difference across the forskolin concentrations relative to the 2% FBS control. A slight decrease in protein concentration was observed at the lower end of the forskolin dose response which would parallel the decreased skeletal α-actin mRNA abundance. The response was not significantly less than the 2% FBS control.


protein concentration ug/ml
1.4
1.2
1
0.8
0.6
0.4
0.2
0
2%
10-8 M FOR
10-7 M FOR
10-6 M FOR
10-5 M FOR
10-4 M FOR

Duration of Treatment on Response to β-adrenergic Agonist

The effect of duration of βAA stimulation with ISO at 10^{-5} M on SKA mRNA abundance in C2C12 myotubes was determined. Abundance of SKA mRNA increased over 1, 4, 8, 24, and 48 hours exposure to ISO reaching maximal levels at 72 hours post treatment. The response at 72 hours was significantly greater than the 2% FBS control ($p<.05$) (Figure 21-22).

β-adrenergic Pathway Component Modulation

Skeletal α-actin mRNA abundance was increased in response to stimulation of C2C12 myotubes with ISO (10^{-5} M). The response to ISO stimulation was abolished by addition of propranolol (PRO) (10^{-4} M) (a non-selective βAR antagonist) in combination with ISO. The reduction in SKA mRNA abudance in response to PRO suggests that the ISO stimulated increase is occurring through the βAR mediated signal transduction cascade.

In contrast to the previously reported dose response study, no increase in SKA mRNA abundance was detected in response to FOR (10^{-4} M). In the same study, however, the addition of cAMP (1 mM) increased levels of SKA mRNA abundance relative to 2% FBS control ($p<.05$)(Figure 23). The lack of a FOR effect may be attributable to inactive FOR in the series of experiments on pathway component modulation.

Inhibition of kinases βAA stimulated signaling pathways would be expected to depress the SKA mRNA response to βAA stimulation, if the increased mRNA abundance is due

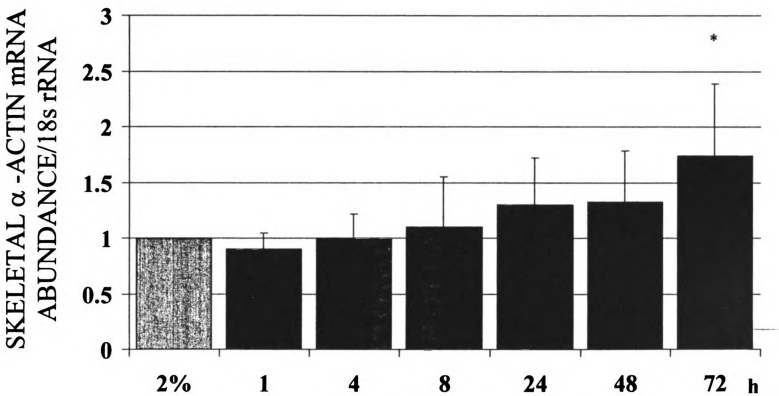
Figure 21 Duration of isoproterenol stimulation on skeletal α-actin mRNA abundance in C2C12 myotubes

Optimal duration of exposure of C2C12 myotubes to isoproterenol (10^{-5} M) for maximal expression of skeletal α-actin mRNA abundance was determined.

Time points included a 2% FBS control, 1 hour exposure to isoproterenol (10^{-5} M), 4 hours exposure to isoproterenol (10^{-5} M), 8 hours exposure to isoproterenol (10^{-5} M), 24 hours exposure to isoproterenol (10^{-5} M), 48 hours exposure to isoproterenol (10^{-5} M), and 72 hours exposure to isoproterenol (10^{-5} M). The treatments were initiated so that all cells across treatments were maintained in culture for the same duration of time. Media was replenished every 24 hours. Treatments at 30 minutes, 1 hour, 4 hours, and 8 hours were prepared in media that was collected from and returned to plates to avoid introducing new media.

Ten micrograms total RNA from C2C12 myotubes were separated on 1.2% agarose denaturing gel, transferred to nylon membrane and hybridized against the 105 bp skeletal α-actin probe. The same blots were stripped and rehybridized with an 18S rRNA probe. Following non-radioactive detection methods, the blots were analyzed using videodensitometry. Data are expressed per unit of 18S rRNA.

Data indicate increased skeletal α-actin mRNA abundance/18S rRNA response to duration of isoproterenol stimulation. The maximal response was determined at 72 hours and was significantly greater than the 2% FBS control ($p<.05$).



*Mean significantly different from 2% FBS control, ($p<.05$).

Figure 22 Duration of isoproterenol stimulation on skeletal α-actin mRNA and 18S rRNA abundance in C2C12 myotubes

Optimal duration of exposure of C2C12 myotubes to isoproterenol (10^{-5} M) for maximal expression of skeletal α-actin mRNA abundance was determined.

Time points incuded (A) 2% FBS control, (B) 1 hour exposure to isoproterenol (10^{-5} M), (C) 4 hours exposure to isoproterenol (10^{-5} M), (D) 8 hours exposure to isoproterenol (10^{-5} M), (E) 24 hours exposure to isoproterenol (10^{-5} M), (F) 48 hours exposure to isoproterenol (10^{-5} M), and (G) 72 hours exposure to isoproterenol (10^{-5} M). The treatments were initiated so that all cells across treatments were maintained in culture for the same duration of time. Media was replenished every 24 hours. Treatments at 30 minutes, 1 hour, 4 hours, and 8 hours were prepared in media that was collected from and returned to plates to avoid not introducing new media.

Ten micrograms total RNA from C2C12 myotubes were separated on 1.2% agarose denaturing gel, transferred to nylon membrane and hybridized against the 105 bp skeletal α-actin probe. The same blots were stripped and rehybridized with an 18S rRNA probe. Following non-radioactive detection methods, the blots were analyzed using videodensitometry. Skeletal α-actin mRNA and 18S rRNA Northern blots are shown.

Data illustrate an inreased skeletal α-actin mRNA abundance over duration of isoproterenol stimulation . No increase in 18S rRNA abundance was observed. The maximal response (skeletal α-actin mRNA/18S rRNA ratio) was determined at 72 hours and was significantly greater than the 2% FBS control ($p<.05$).

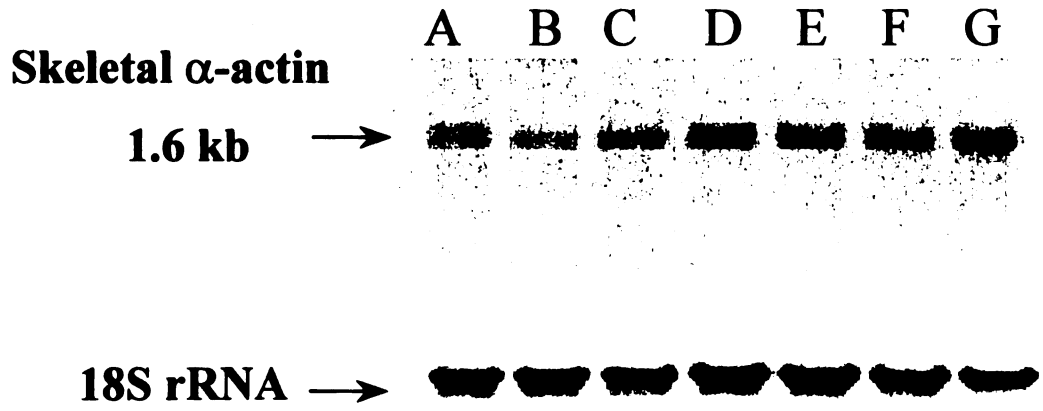

A B C D E F G
Skeletal α-actin
1.6 kb
18S rRNA

Figure 23 β-adrenergic pathway component modulation and skeletal α-actin mRNA abundance in C2C12 myotubes

C2C12 myotubes were treated with modulators of key components of the β-adrenergic signal transduction pathway. Depicted are the skeletal α-actin mRNA/18S rRNA ratios following treatment with a series of modulators for 48 hours. The diagram represents the following treatments (1) 2% FBS control, (2) isoproterenol, 10^{-5} M, (3) isoproterenol, 10^{-5} M and propranolol, 10^{-4} M, (4) cAMP, 1 mM, (5) forskolin, 10^{-4} M, (6) isoproterenol, 10^{-5} M and HA1004, 75 mM (7) isoproterenol, 10^{-5} M and H7, 100 mM.

Ten micrograms total RNA from C2C12 myotubes were run on a 1.2% agarose denaturing gel, transfered to nylon membrane and hybridized against the 105 bp skeletal α-actin probe. The same blots were stripped and rehybridized with an 18S rRNA probe. Following non-radioactive detection methods, the blots were analyzed using videodensitometry. Data are expressed per unit of 18s rRNA.

Results indicate a significant increase in skeletal α-actin mRNA abundance above 2% FBS controls ($p<.05$) upon stimulation of C2C12 myotubes with isoproterenol. In addition, cAMP, a key second messenger in the beta-adrenergic pathway, increased skeletal α-actin mRNA abundance above 2% FBS controls ($p<.05$). Kinase inhibition by HA1004 or H7 indicated a decreased skeletal α-actin mRNA abundance upon isoproterenol stimulation. Skeletal α-actin mRNA abundance was significantly lower ($p<.05$) in those samples treated with isoproterenol and the specific kinase inhibitor relative to isoproterenol alone indicating that the inhibited kinase was involved in the isoproterenol response.

Data support the observation that isoproterenol stimulated increases in skeletal α–actin mRNA abundance in C2C12 cells is occurring though a beta-adrenergic receptor mediated cAMP-dependent cascade of events.

SKELETAL α -ACTIN mRNA
ABUNDANCE/18s rRNA
1.4
1.2
1
0.8
0.6
0.4
0.2
0
2%
ISO
ISO/P
CAMP
FORS
ISO/HA
ISO/H7
ISO>2% P<0.05
CAMP>2% P<0.05
ISO>ISO/HA P<0.05
ISO>ISO/H7 P<0.05

in part to that kinase activity. ISO stimulated SKA mRNA abundance was returned to control levels by inhibiting PKA with the PKA inhibitor, HA1004 (75 mM). A potent inhibitor of both PKA and PKC, H7 (100 mM), also depressed the SKA mRNA abundance response to ISO treatment. Inhibition of PKC was attempted with a potent PKC inhibitor, staurosporine; however, the chemical appeared to be toxic to cells. The results observed using staurosporine at concentrations ranging from 25 ng/ml to 75 ng/ml suggest a toxic effect rather than a specific inhibition of PKC based on the observation that cells exhibited approximately 44% less expression of the 18S rRNA to that of 2% FBS controls. The major decline in 18S rRNA abundance and ravaged appearance of cells indicated that cell death was occurring. Staurosporine has been reported to cause apoptosis in cells (Sigma data sheet, Sigma Chemical Co., St. Louis, MO). Results utilizing protein kinase inhibitors indicate that inhibition of PKA upon βAA stimulation blocks the documented increase in βAA stimulated SKA mRNA abundance. Experiments attempting to specifically inhibit PKC during βAA stimulation are inconclusive. Data using a PKA/PKC inhibitor led to a less intense depression in βAA stimulated SKA mRNA abundance than inhibition of PKA alone which may suggest that the inhibitor is not as potent in inhibiting PKA as HA1004 (Figure 23).

These data suggest that the βAA-stimulated increase in SKA mRNA abundance in C2C12 myotubes is occuring via a βAR-mediated, PKA dependent pathway. Data presented here are contrary to adrenergic stimulation of SKA mRNA abundance in cardiac myocytes. Bishopric et al., (1982) reported that the increase in SKA mRNA abundance

in cardiac myocytes may be occuring through either an α or β adrenergic receptor mediated pathway and independent of cAMP/PKA. The results support the notion of different pharmacological properties of agonists in different tissues. The difference may lie in the fact that different tissues may express more than one type of adrenergic receptor subtype. Mills and Mersmann (1995) confer that it is not clear how a cell with multiple βAR subtypes integrates the function of these receptor subtypes.

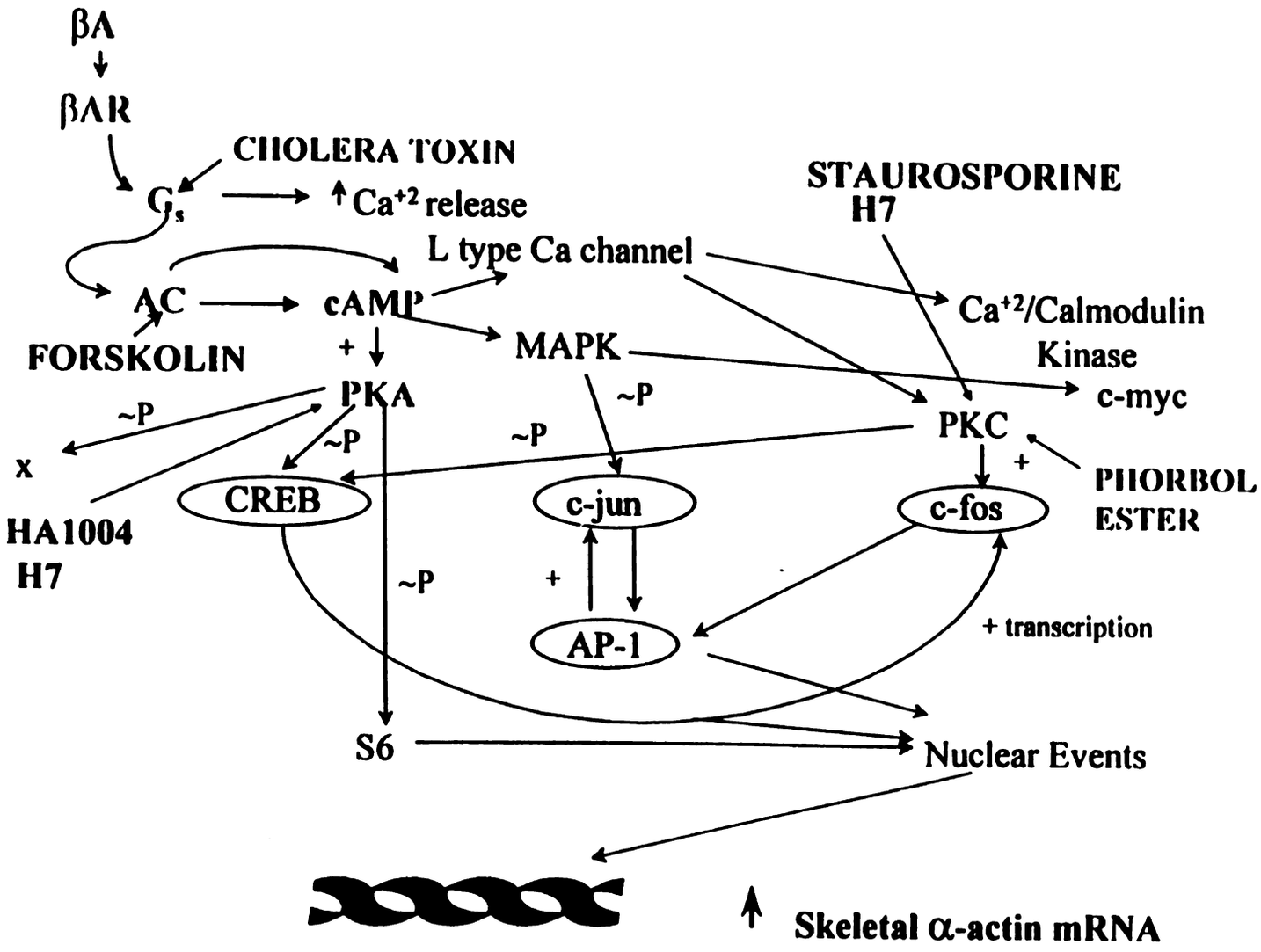
While βAA-stimulation of C2C12 myotubes leads to increased expression of SKA mRNA abundance the addition of a βAR antagonist, PRO or PKA inhibitor results in a depressed response to βAA stimulation. The data suggest that βAA-stimulated increases in SKA mRNA abundance are cAMP-dependent and involve activation of PKA-mediated events in C2C12 myotubes. Involvement of other signaling pathways in the βAA-stimulated increase in SKA mRNA abundance remain unclear (Figure 24).

Figure 24 Summary figure-Chapter 2

Chapter 2 addressed the effect of β-adrenergic stimulation on skeletal α-actin mRNA abundance in C2C12 myotubes. In review: stimulation at the β-adrenergic receptor by isoproterenol (10^{-5} M) resulted in increased skeletal α-actin mRNA abundance relative to the 2% FBS control. Furthermore, the addition of cAMP (1 mM) or forskolin (10^{-4} M) also significantly increased skeletal α–actin mRNA abundance above the 2% FBS control. The increase in skeletal α-actin mRNA abundance resulting from β-adrenergic stimulation by isoproterenol was abolished by the addition of propranolol (10^{-4} M) or HA1004 (75 mM). Results indicate that the increased skeletal α-actin mRNA abundance observed upon β–adrenergic stimulation of C2C12 myotubes is occurring through a cAMP dependent protein kinase pathway.

CHAPTER 3

β -ADRENERGIC STIMULATION INCREASES PHOSPHORYLATION OF A ~90 kDa PROTEIN IN C_2C_{12} MYOTUBES


βA
βAR
CHOLERA TOXIN
G_s
↑Ca^{+2} release
STAUROSPORINE
H7
L type Ca channel
AC
cAMP
Ca^{+2}/Calmodulin
Kinase
FORSKOLIN
+
MAPK
c-myc
PKA
~P
PKC
x
HA1004
H7
CREB
c-jun
c-fos
+
PHORBOL
ESTER
AP-1
+ transcription
S6
Nuclear Events
Skeletal α-actin mRNA

Abstract

β-adrenergic agonist stimulation via G_s protein results in the elevation of intracellular cAMP and subsequent activation of cAMP-dependent protein kinase, PKA, a serine/threonine kinase which is capable of phosphorylating a wide array of proteins. The objective was to determine if the response of differentiated C2C12 muscle cells to βAA stimulation was manifested by an increase in protein phosphorylation. An apparent increase in phosphorylation of an ~ 90 kDa protein was observed in response to increasing concentrations of isoproterenol (ISO). Exposure of C2C12 myotubes to a combination of ISO and a phosphodiesterase inhibitor (PDE-I) (10 μM) enhanced the phosphorylation of the ~ 90 kDa protein. Further investigation indicated an increased phosphorylation of the ~90 kDa protein in response to dbt-cAMP (1 mM), FOR (10^{-4} M) and CTOX (500 ng/ml). Responses observed appear to be mediated by an increase in protein phosphorylation rather than an increase in protein synthesis, since cyclohexamide (CHX) (10 μg/ml) did not alter the response observed in treated cells. Anti-P-threonine. immunoblots of extracts of cells at progressive stages of development revealed that appearance of the ~90 kDa phosphoprotein followed a developmental pattern of expression which paralleled that of sarcomeric myosin (SM). Further characterization of the protein via cell fractionation revealed the protein to be cytoplasmic rather than nuclear or myofibrillar. These results indicate the presence of a cytoplasmic, ~90 kDa

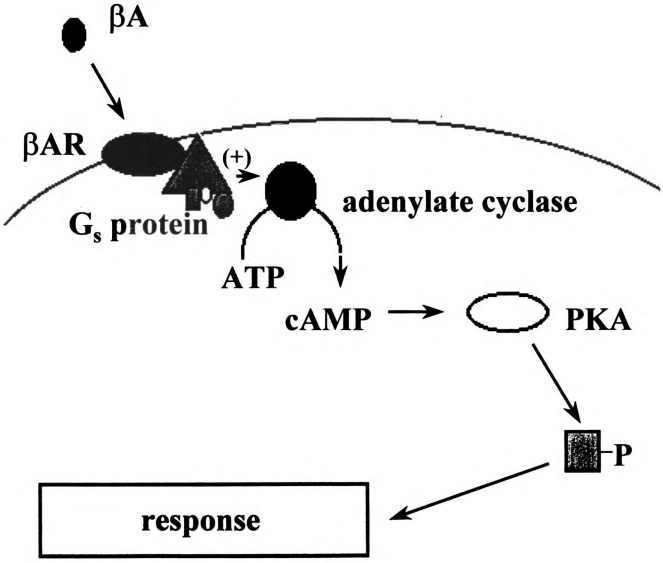
phosphoprotein that is phosphorylated in response to βAA stimulation in C_2C_{12} myotubes.

Introduction

Skeletal muscle is the primary target tissue for enhanced protein accretion in animals fed βAA (Bergen et al., 1996, Skjaerlund et al., 1993, Paterson et al., 1984, Gordon et al., 1984). βAA's also stimulate protein synthesis and accretion in cultured muscle cells (Bergen et al., 1996, Shani et al., 1981, Anderson et al., 1990). The mechanism by which βAA influence protein accretion in muscle remains unresolved. Extracellular signals regulate gene expression by triggering signal transduction cascades that result in the modulation of transcription factor activity. Signal transduction cascade gene activation is most commonly achieved by changes in the phosphorylated state of nuclear proteins. Phosphorylation affects transcription factor activity at several distinct levels. It can modulate their intracellular localization by controlling the association with other proteins, have both negative and positive effects on their binding activity, and modulate the activity of their transcriptional activation domains. In addition to phosphorylation, protein-protein interactions also have an important role in mediating a cross-talk at the nuclear level between different signaling pathways (Karin, 1991) (Figure 1). The proposed mechanism for βAA induced muscle protein accretion is through a G_s-protein linked cAMP-dependent protein kinase cascade (Bergen et al., 1991, Bowman et al., 1969) (Figure 25). Most aspects of adrenergic stimulation do not ordinarily require an increase in the synthesis of new proteins. Post-receptor events may involve a complex

Figure 25 Classical β-adrenergic signal transduction pathway

The illustration depicts the classical β-adrenergic signal transduction pathway. Stimulation of the signaling cascade is initiated by binding of a β-adrenergic agonist at the membrane-bound β-adrenergic receptor. Binding at the receptor causes dissociation of the G-stimulatory protein α-subunit which activates the enzyme adenylate cyclase. Adenylate cyclase catalzyes the conversion of intracellular ATP to the second messenger, cAMP. cAMP is then capable of activating the cAMP dependent protein kinase, protein kinase A. The activated protein kinase A dissociates into a regulatory and active catalytic subunits. The catalytic subunits are then capable of phosphorylating a wide array of proteins within the cell eliciting the β-adrenergic stimulated response.


βA
βAR
(+)
G_s protein
adenylate cyclase
ATP
cAMP
PKA
P
response

interplay among various proteins by covalent modification through phosphorylation /dephosphorylation (Iwaki et al., 1990). Protein phosphorylation regulates a diverse range of cellular responses (Meek et al., 1992). Although many of these regulated proteins have been identified and characterized in other systems, many newly discovered or yet undiscovered proteins may play a pivotal role in the biochemical mechanism behind βAA induced muscle protein accretion (Iwaki et al., 1990, Meek et al., 1992). Identification of skeletal muscle proteins phosphorylated in response to βAA is essential for understanding the biochemical mechanism behind βAA-induced muscle protein accretion (Beermann et al., 1986).

The objective is to identify proteins phosphorylated in response to βAA stimulation in C2C12 myotubes. It is anticipated that the proteins identified represent a phospho-protein involved in the βAA hypertrophic response.

Materials and Methods

Cells

C_2C_{12} muscle cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Approximately 50,000 cells/dish were seeded into 60 mm diameter dishes in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO Inc., Grand Island, NY) containing 10% fetal bovine serum (FBS) (Sigma Chemical Co., St. Louis, MO). Cells were grown to confluence in DMEM containing 10% FBS in a humidified atmosphere of 5% CO_2 and 95% air at 37° C. Upon confluence, growth media was replaced with differentiation media (DMEM, 2% FBS) to stimulate myotube formation. Medium was replaced every 48 hours unless otherwise indicated during the experiments.

Sample Preparation

Myotube cultures were rinsed 3 times in phosphate buffered saline, harvested using a low salt NFEP homogenization buffer (.05 M NaF, .01 M EDTA, .075 M NaCl, .015 M Na_2HPO_4, pH 7.0), and sonicated using a Branson Sonic Power Co., Sonifier-cell disrupter 350 at setting #4 for approximately 5 seconds. Aliquots of each sample were saved for protein and DNA quantification, and for SDS-PAGE. Protein was determined using the method of Bradford (Bradford, 1976) using commercial reagents (Bio-Rad,

Hercules, CA). DNA was determined fluorometrically using the Hoescht 33258 reagent as described by West (1985). Sample aliquots for SDS-PAGE were prepared by mixing equal portions of the aliquot and hot 2x treatment buffer (125 mM Tris, 4% SDS, 20% glycerol, 10% MCE, pH 6.8). Samples not used immediately were stored at -80° C and heated for 5 minutes at 95°C before loading onto gels.

SDS-PAGE and Western Blotting

Samples were loaded on an equal protein basis on an 8% acrylamide (37.5:1) separating gel with a 4% acrylamide stacking gel and fractionated at 165 volts for approximately 1 hour. Proteins were electrophoretically transferred to Immobilon P membrane (Millipore, Inc., Bedford, MA) at 100 V for 1 hour. Membranes were allowed to air dry and stored until blotting. Initially, membranes were blocked for 1 hour in blocking buffer (1% BSA, .05% Tween 20 in Tris-buffered saline). Following blocking, blots were incubated with either a rabbit anti-P-thr. (#61-8200) (.5 ug/ml) or anti-P-ser. (#61-8100) primary antibody (Zymed Technologies, Inc., San Francisco, CA). Antibodies were titrated for optimal concentration for use in C2C12 myotube extracts. Following incubation with primary Ab, the blots were incubated with an anti-rabbit secondary antibody conjugated to either alkaline phosphatase (AP) or horseradish peroxidase (HRP) (1:20,000) (Sigma Immunochemicals, St. Louis, MO). Membranes were washed three times (5 minutes each) in blocking buffer following each Ab incubation. Antibody binding was visualized by exposure of membranes to colorimetric AP reagent (BCIP/NBT; Bio-Rad, Hercules,

CA) or chemilluminescent HRP substrate (Super-Signal Chemilluminescent Substrate for Western Blotting; Pierce, Rockford, IL).

Two-Dimensional (2-D) Gel Electrophoresis

2-D gel electrophoresis was utilized to further identify the responsive protein relying on separation by the protein's isoelectric point (pI) and molecular weight. 20 ul aliquots of the cytoplasmic fraction of cell extracts were applied to 3.5% tube gels cross-linked with piperazine diacrylamide containing 9M urea and 2% Bio-Lyte ampholytes (1 part 3/10 and 2 parts 5/7) according to manufacturers instructions (Bio-Rad, Hercules, CA). The tube gel was run on a 8% acrylamide (37.5:1) slab gel for further fractionation by molecular weight. Estimation of the unknown proteins pI was determined through comparison to 2-D standards (Bio-Rad, Hercules, CA) run in parallel to the cytoplasmic fraction sample.

Duration of β-adrenergic Stimulation on Protein Phosphorylation

C2C12 myotubes were stimulated with the βAA, ISO (10^{-5} M) for various lengths of time to determine the time of maximal phosphorylation in response to ISO stimulation. Cell maintenance was performed as described earlier. Media were replaced every 24 hours during treatment. Treatments were initiated 48 hours prior to harvest and continued through 30 minutes prior to harvest. Treatments were initiated in this fashion to maintain all cells in culture for the same amount of time and to minimize the confounding effects of fresh media at later time points. Treatment at time points 8, 4, 1 hour and 30 minutes

were prepared by removing media from cells, adding ISO to the media, and redistributing this media to plates so new media was not introduced at those time points. The control samples were maintained in DMEM + 2% FBS for the 48 hour duration of the study.

Phosphodiesterase Inhibitor

β-adrenergic stimulation increases intracellular cAMP. The enzyme phosphodiesterase (PDE) breaks down cAMP, thereby eliminating the βAA induced response. Treatment with a phosphodiesterase inhibitor (PDE-I) (4-(3-butoxy-4-methoxybenzyl)-imidizolidin-2-one) (Sigma Chemical Co., St. Louis, MO) in a βAA-responsive system should result in increased levels of intracellular cAMP and a potentiated/enhanced response to βAA stimulation. C2C12 myotubes were pre-incubated with a PDE-I (1 ug/ml) for 1 hour prior to and during stimulation with ISO for either 30 minutes or 1 hour before harvesting as described above.

Cyclohexamide Treatment

The effects of βAA stimulation are usually rapid in onset, often occurring within minutes of agonist binding, and do not ordinarily require an increase in the synthesis of new proteins. Treatment of C2C12 myotubes with cyclohexamide (CHX) (Sigma Chemical Co., St. Louis, MO) would indicate if the response to ISO stimulation was due to the synthesis of new proteins or an increased phosphorylation of the protein of interest. C2C12 myotubes were pre-incubated with CHX (1 μg/ml) for one hour prior to and during stimulation with ISO for either 30 minutes or 1 hour before harvesting as

described above. The time points chosen, 1 hour or 30 minutes, represent time points that are rather early after stimulation to see a phosphorylation response. Therefore, an increased phosphorylation response observed at either time point would strongly indicate that the response observed was due to phosphorylation rather than increased protein synthesis.

Cell Fractionation

Cells were rinsed 3x in NFEP buffer, scraped with a rubber policeman, and Dounce homogenized. Samples were centrifuged at 1,000 x g for 15 minutes. The supernatant was saved and represented the cytoplasmic fraction while the pellet was resuspended in NFEP buffer and represented the myofibrillar and nuclear fractions. Further fractionation of the sarcoplasmic fraction was achieved by centrifugation of the sarcoplasmic fraction at 100,000 x g for 1 hour to pellet the membrane fraction. The sample was washed once with NFEP buffer and recentrifuged at 100,000 x g for an additional hour. Aliquots of each sample were analyzed by protein and DNA assay and by SDS-PAGE as described above.

Statistical Analysis

Data were analyzed through analysis of variance by using the program of SAS (SAS, 1996). Differences among means were tested for significance ($P<.05$) using the method of Tukey or by comparison across treatments by contrast.

Results and Discussion

Phospho-Amino Acid Profiles

Initially, cell extracts of untreated mytotubes were prepared as described earlier and immunoblotted using either an anti-P-thr or anti-P-ser primary Ab in an effort to determine what the phosphoprotein profile for each antibody looked like. The anti-P-thr Ab labeled a greater number and overall intensity of bands relative to the anti-P-ser Ab (Figure 26) which revealed only light labeling of a few phospho-proteins (Figure 26). We then chose to examine the effect of βAA stimulation on C_2C_{12} myotube cell extracts in comparison to control cell extracts in order to identify any potential phosphorylation responses in the protein profile. Cell extracts were prepared and blotted against the anti-P-thr Ab in an attempt to identify any responsive protein/s.

Identification of a β-adrenergic Responsive Protein

Initially, myotubes were stimulated with two concentrations of ISO (10^{-6} M and 10^{-8} M) as well as two concentrations of PDE-I (10 uM or 100 uM). An ~90 kDa protein appeared to be phosphorylated in response to βAA stimulation by ISO (10^{-6} M) (Figure 27) as determined by an increased band intensity relative to the 2% FBS control. Although other bands appeared that may have shown an increased phosphorylation response to βAA stimulation, the intensity at ~90 kDa suggested that the ~90 kDa protein may be

Figure 26 Anti-P-serine and anti-P-threonine immunoblot of C2C12 myotube cell extracts

Ten micrograms total protein from untreated C2C12 myotube cell extracts were run on 8% PAGE, transfered to nylon membrane and immunoblotted using either an anti-P-serine (0.5 μg/ml) or anti-P-threonine (0.5 μg/ml) primary antibody. The secondary Ab was an anti-rabbit IgG conjugated to alkaline phosphatase. Detection was accomplished through a colorimetric procedure.

Data indicate a predominance of intense bands in the anti-P-threonine immunoblot.

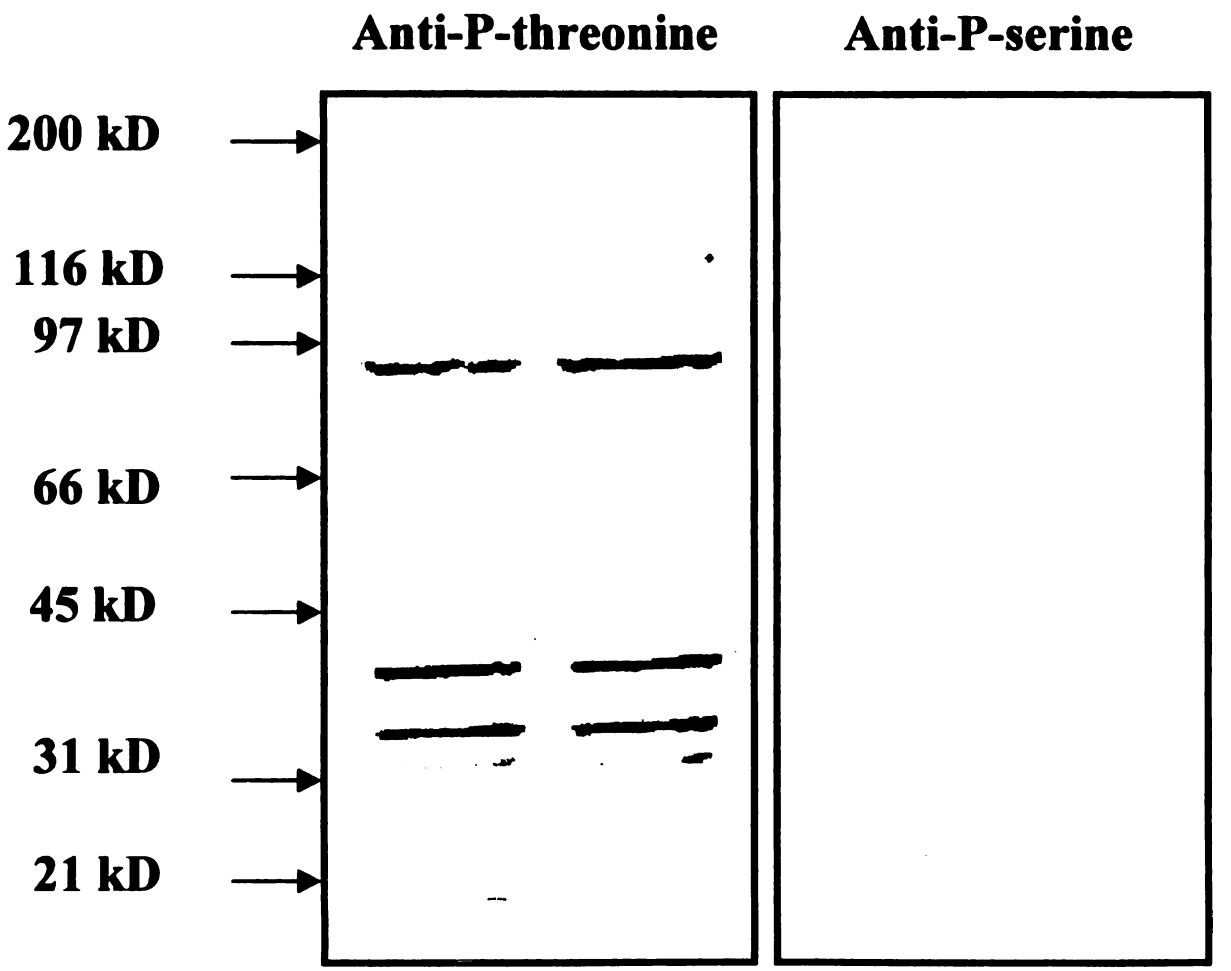
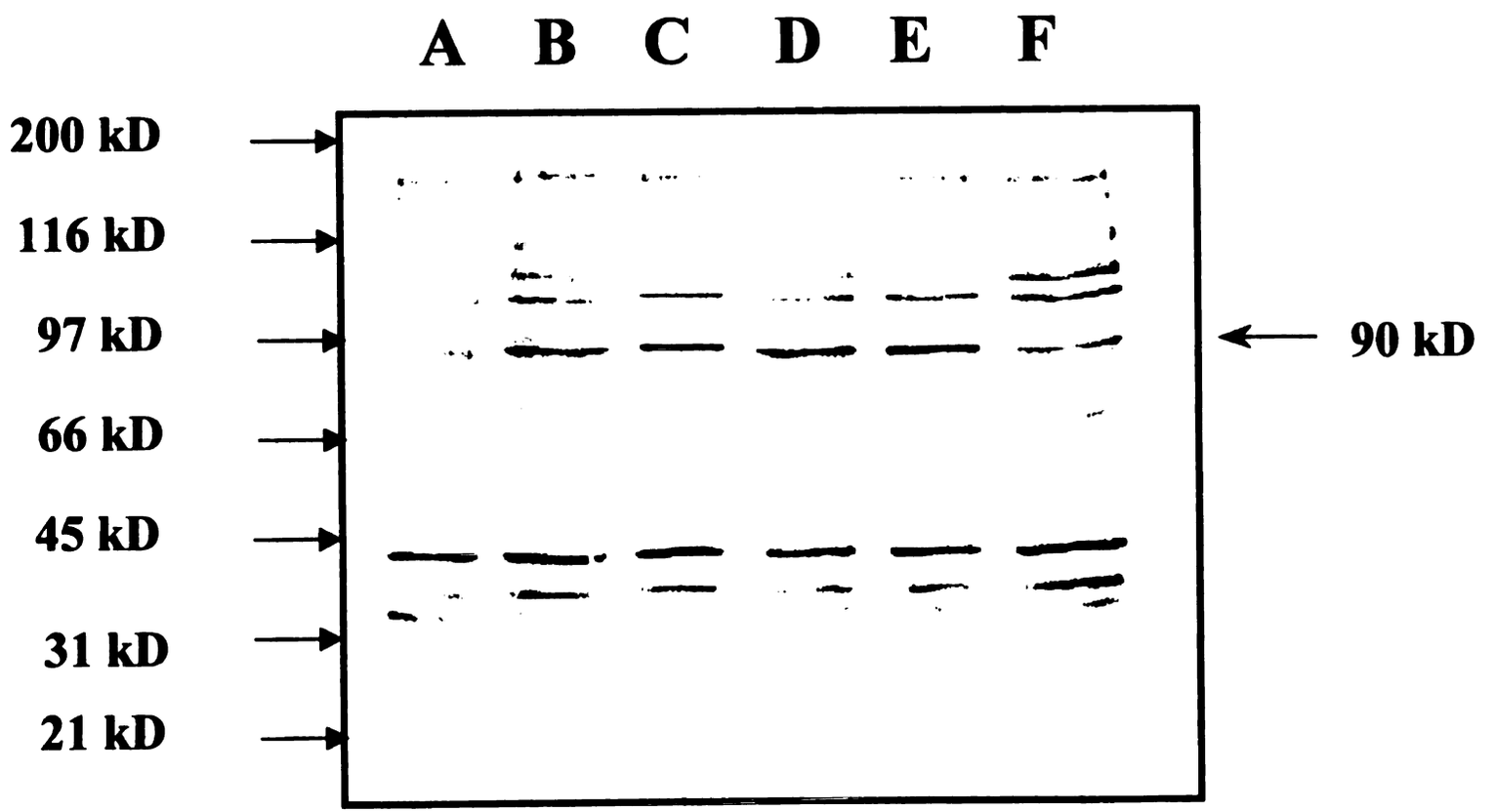

Anti-P-threonine
Anti-P-serine
200 kD
116 kD
97 kD
66 kD
45 kD
31 kD
21 kD

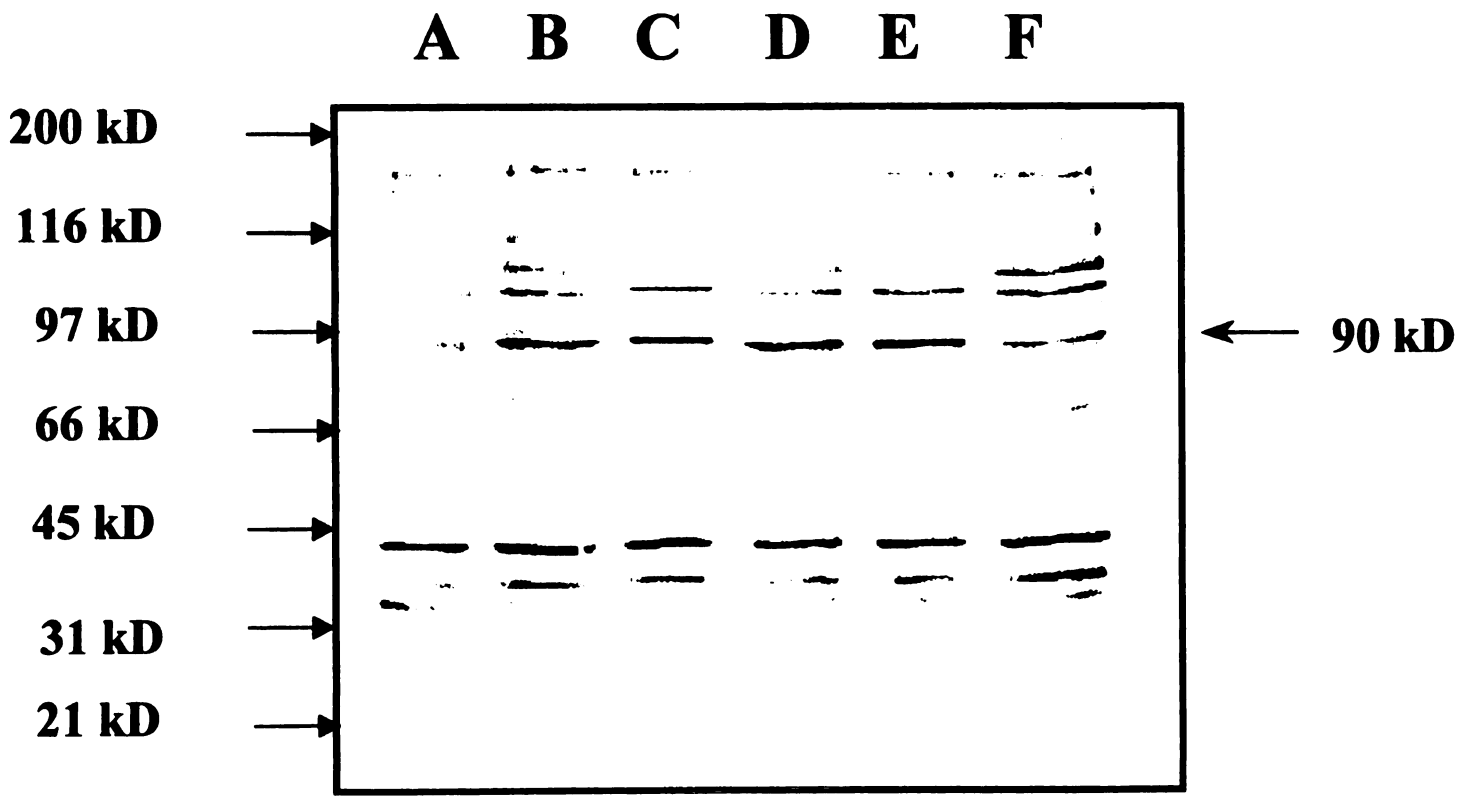
Figure 27 Identification of a ~90 kDa β-adrenergic responsive protein in C2C12 myotube cell extracts

Ten micrograms total protein from treated and control C2C12 myotube cell extracts were run on 8% PAGE, transfered to nylon membrane and immunoblotted using an anti-P-threonine primary antibody. Lanes represented: (A) 2% FBS control, (B) isoproterenol, 10^{-8} M, (C) isoproterenol, 10^{-6} M, (D) isoproterenol, 10^{-6} M + 1 μg/ml phosphodiesterase inhibitor, (E) isoproterenol, 10^{-6} M + 10 μg/ml phosphodiesterase inhibitor, (F) 10% FBS control.

The anti-P-threonine immunoblot reveals a greater intensity relative to control protein profile at a band ~90 kDa. The intensity of the band at ~90 kDa is appears more intense at each concentration of isoproterenol and increases slightly in the presence of a phosphodiesterase inhibitor relative to the 2% FBS control.

The response to (1) isoproterenol and (2) isoproterenol/phosphodiestease inhibitor treatments at either concentration suggests that the ~90 kDa protein is a likely candidate to investigate β-adrenergic regulated phosphorylation in C2C12 myotubes. The phosphorylation response appears to be induced by the addition of a β-adrenergic agonist and slightly intensified by the inhibition of regulatory enzyme.


A B C D E F
200 kD
116 kD
97 kD
66 kD
45 kD
31 kD
21 kD
90 kD


A B C D E F
200 kD
116 kD
97 kD
66 kD
45 kD
31 kD
21 kD
90 kD

more abundant and therefore an easier candidate to study than proteins at lower band intensities. An apparent increase in phosphorylation of a ~ 90 kDa protein in response to either concentration of ISO and a slight increase in phosphorylation response to either concentration of PDE-I was detected. The response observed indicates that βAA stimulation through the βAR by ISO can be potentiated by the addition of a PDE-I. The data suggest that ISO stimulated phosphorylation is the result of a cAMP linked cascade in C2C12 myotubes. The phosphorylation of the ~90 kDa protein appears to be dependent on a cAMP regulated cascade mechanism most likely through the activation of protein kinase A (PKA).

Pathway Component Modulation

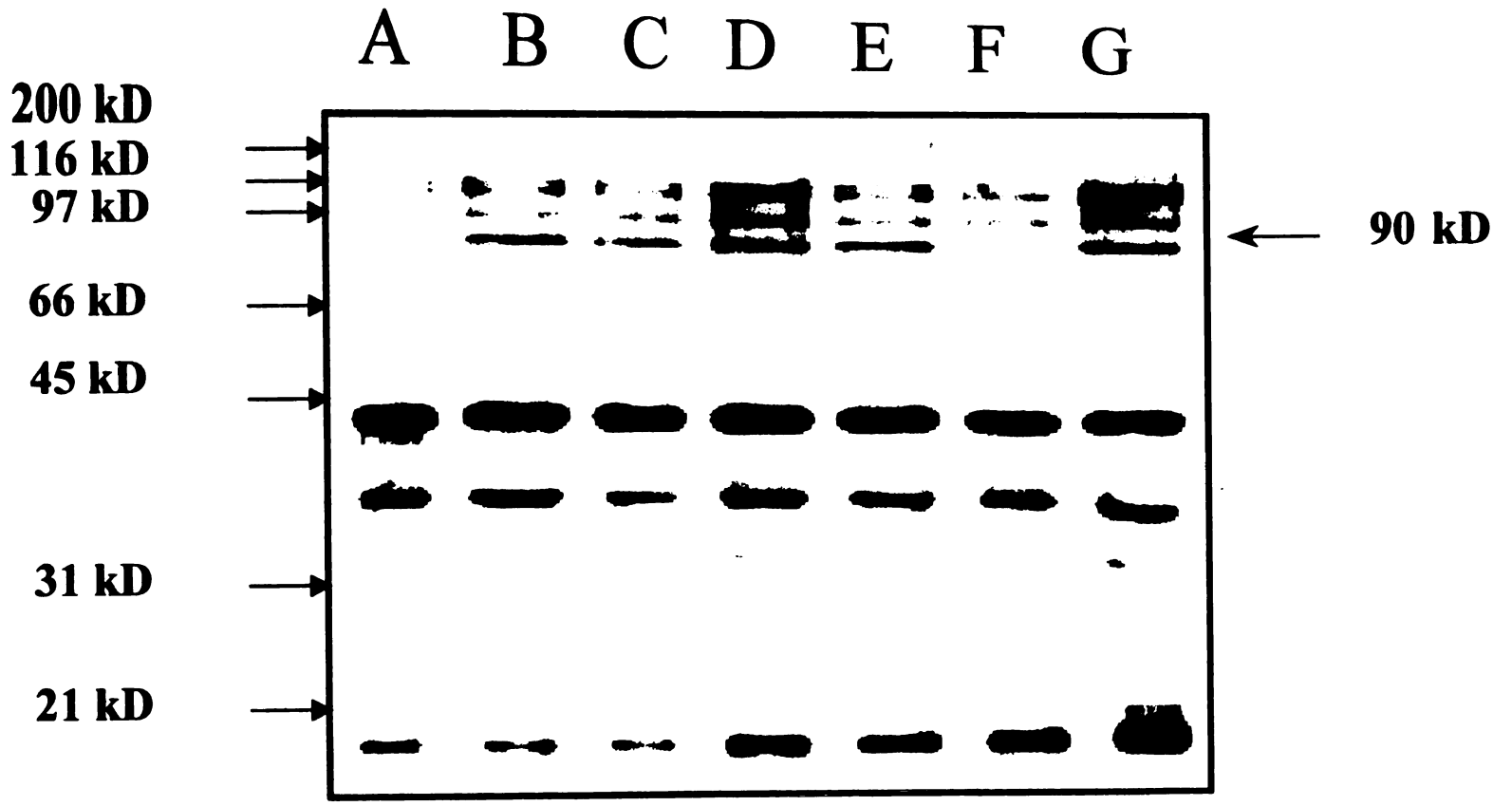
Further investigation into the mechanism of the phosphorylation of the ~90 kDa protein was accomplished by targeting direct components within the proposed βAA pathway. FOR (10^{-4} M), ISO (10^{-5} M), RAC (10^{-6} M), cAMP (1mM), PE (2 μM), and CTOX (500 ng/ml) were administered to C2C12 myotubes for 48 hours. At 48 hours, the cells were harvested in NFEP buffer and cell extracts prepared as described above. Based on densitometric scans, an approximate three-fold increase in intensity of an anti-P-thr labeled band above 2% FBS control myotube extracts was observed through stimulation by components of the βAA pathway. No stimulation occurred with the PE (Figure 28).

Figure 28 Anti-P-threonine immunoblot against β-adrenergic pathway component stimulated C2C12 myotube cell extracts

Ten micrograms total protein from treated or control C2C12 myotube cell extracts were run on 8% PAGE, transfered to nylon membrane and immunoblotted using an anti-P-threonine primary antibody. Lanes represented: (A) 2% FBS control, (B) forskolin, 10^{-4} M, (C) isoproterenol, 10^{-5} M, (D) ractopamine, 10^{-6} M, (E) cAMP, 1mM, (F) phorbol ester, .5 μM, (G) cholera toxin, 500 ng/ml.

The anti-P-threonine immunoblot illustrates an approximately threefold increase in intensity at ~90 kDa in response to β-adrenergic pathway component modulators relative to 2% FBS control. The intensity determined at ~90 kDa in phorbol ester treated C2C12 myotube extracts was as intense as the 2% FBS control sample.

The data support the observation that the phosphorylation of an ~90 kDa protein in C2C12 myotubes by β-adrenergic agonist stimulation is occurring through the classical β-adrenergic signal transduction pathway.


A B C D E F G
200 kD
116 kD
97 kD
66 kD
45 kD
31 kD
21 kD
90 kD

Duration of β-adrenergic Stimulation on Phosphorylation Response

The duration of exposure of C2C12 myotubes to βAA stimulation on the observed phosphorylation response of the ~90 kDa protein was examined. Considering a phosphorylation response would most likely occur much earlier than any effect on protein abundance. It seemed likely that the optimal response is at a time point earlier than 48 h. Data indicate an increased phosphorylation response to βAA stimulation as early as 30 minutes. A significant response above control extracts was observed at 4 h (Figure 29). The fact that the maximal response is rather late in relation to βAA stimulation may suggest that activation at the βAR may be influencing events in other pathways or other cell compartments which would not be recognized as early as a direct βAA response, or that the response requires protein synthesis.

Verification of a Phosphorylation Response

The ~P response in C2C12 myotubes at 30 minutes and 1 hour was enhanced with inclusion of a PDE-I (10 μM) in addition to ISO as compared to ISO or 2% FBS control (Figure 30). The enhanced response with the PDE-I supports the idea that phosphorylation of the ~90 kDa protein is occrring via a βAR mediated cAMP-dependent pathway. Presumably, increased cAMP from deactivation the PDE activates PKA which may be capable of increased phosphorylation. Cyclohexamide (1 μg/ml) inhibition of protein synthesis did not affect the phosphorylation response to ISO stimulation of C2C12 myotubes at either 30 minutes or 1 hour. Data suggest that the response observed at ~90 kDa in response to ISO/CHX is the result of increased ~P rather than increased protein

Figure 29 Anti-P-threonine immunoblot of isoproterenol stimulated C2C12 myotube cell extracts over time

Ten micrograms total protein from treated or control C2C12 myotube cell extracts exposed to isoproterenol for various time intervals were run on 8% PAGE, transfered to nylon membrane and immunoblotted using an anti-P-threonine primary antibody.
Lanes represented: (A) 2% FBS control, (B) 30 minutes exposure to isoproterenol, 10^{-5} M, (C) 1 hour exposure to isoproterenol, 10^{-5} M, (D) 4 hours exposure to isoproterenol, 10^{-5} M, (E) 8 hours exposure to isoproterenol, 10^{-5} M, (F) 24 hours exposure to isoproterenol, 10^{-5} M, (G) 48 hours exposure to isoproterenol, 10^{-5} M.

The treatments were initiated so that all cells across treatments were maintained in culture for the same duration of time. Media was replenished every 24 hours. Treatments at 30 minutes, 1 hour, 4 hours, and 8 hours were prepared in present media and returned to plates as to not introduce new media.

Data indicate an enhanced response to duration of isoproterenol stimulation as early as 30 minutes with significance above 2% FBS control at 4 hours ($n=4$) ($p<.05$).

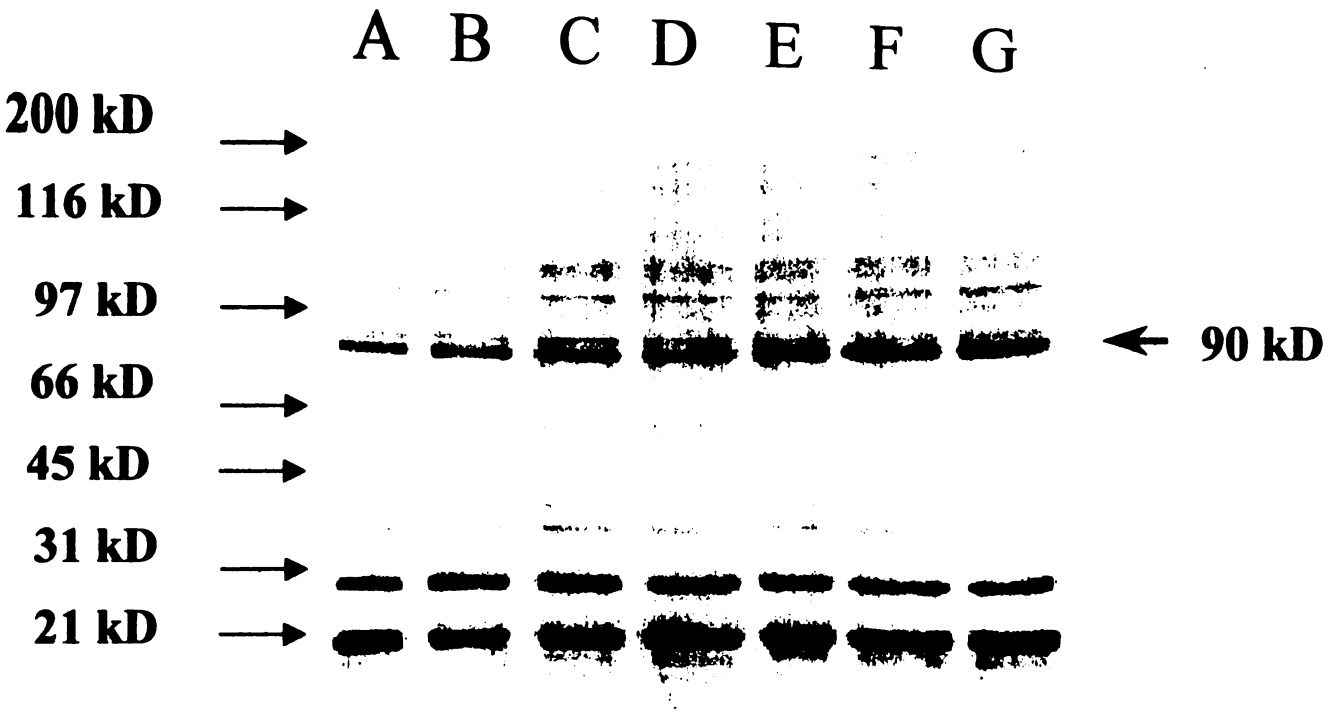
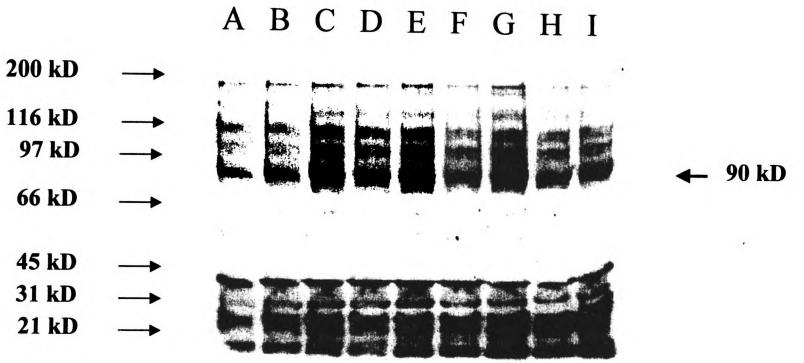

A B C D E F G
200 kD
116 kD
97 kD
66 kD
45 kD
31 kD
21 kD
90 kD

Figure 30 Verification of a phosphorylation response to isoproterenol stimulation in C2C12 myotube cell extracts

Ten micrograms total protein from treated and control C2C12 myotube cell extracts were run on 8% PAGE, transfered to nylon membrane and immunoblotted using an anti-P-threonine primary antibody. Lanes represented: (A) 2% FBS control, (B) isoproterenol, 10^{-5} M at 30 minutes, (C) isoproterenol, 10^{-5} M, + phosphodiesterase inhibitor, 10 μM, at 30 minutes, (D) isoproterenol, 10^{-5} M, at one hour, (E) isoproterenol, 10^{-5} M, + phosphodiesterase inhibitor, 10 μM, at 1 hour, (F) isoproterenol, 10^{-5} M, at 30 minutes, (G) isoproterenol, 10^{-5} M + cyclohexamide, 1 μg/ml, at 30 minutes, (H) isoproterenol, 10^{-5} M, + cyclohexamide, 1 μg/ml, at 1 hour, (I) isoproterenol, 10^{-5} M, at 1 hour.

Data presented support increased phosphorylation rather than an increase in amount of phosphoryated protein. Data suggest an increased phosphorylation in response to the addition of a phosphodiesterase inhibitor, suggesting increased phosphorylation via the signal transduction pathway due to increased intracellular cAMP and therby increased PKA activity at each time point. No substantial evidence exists to support the notion of increased protein involved in the response at ~90 kDa.


A B C D E F G H I
200 kD
116 kD
97 kD
66 kD
45 kD
31 kD
21 kD
90 kD

synthesis (Figure 30). Considering a phosphorylation response is observable within 1 h suggests that the response is due to protein phosphorylation rather than an increase in newly synthesized protein.

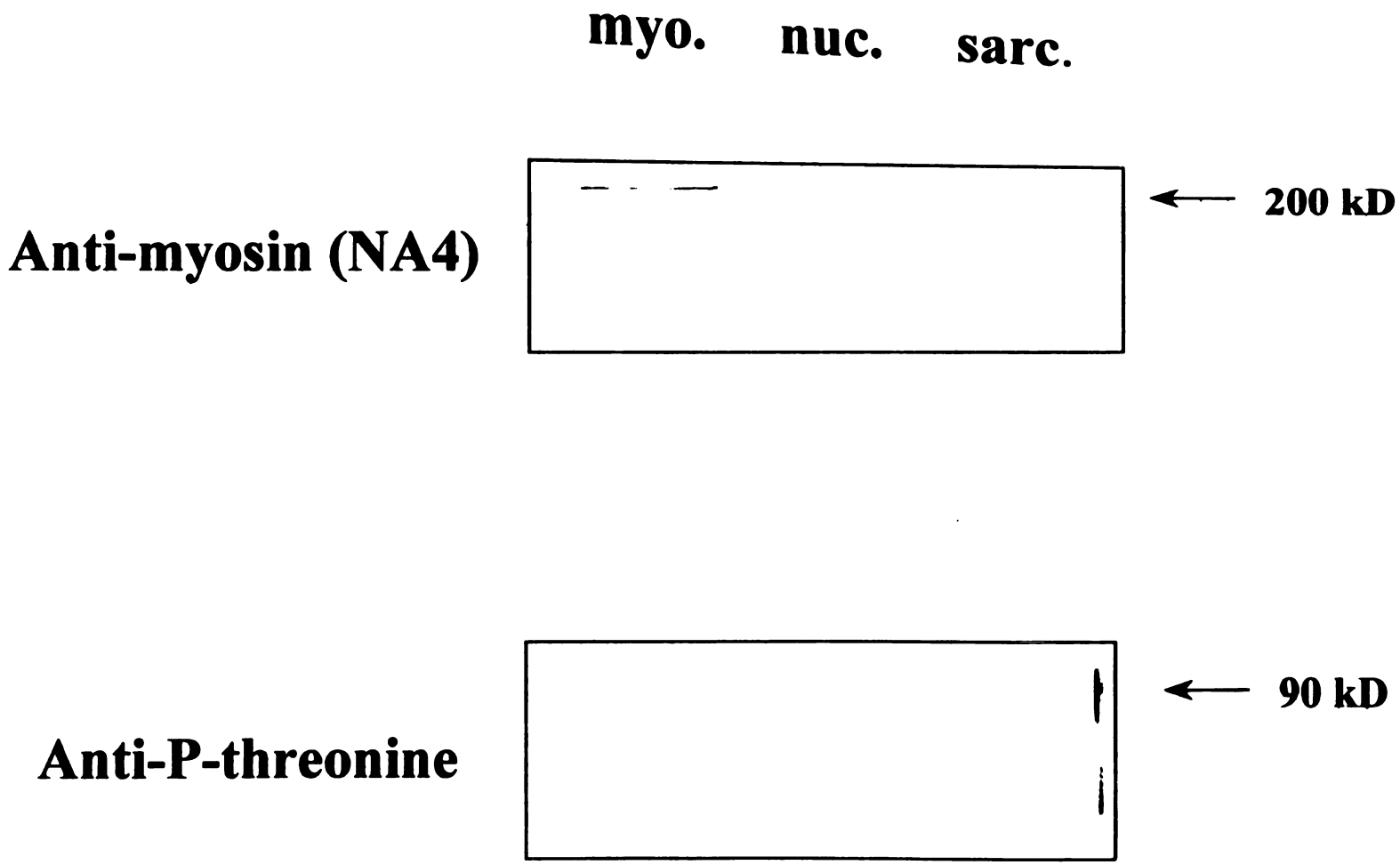
Cell Fractionation

Initially, we were hoping to identify a phospho-protein involved in myofibrillar gene expression. Determining the cellular fraction in which the ~90 kDa protein was most predominant would aid in further identification of the protein. Cellular fractionation was accomplished as described above. We were unable to successfully isolate a pure nuclear fraction most likely due to rupture of nuclear membranes during Dounce homogenization. We had, however, two distinct fractions: a myofibrillar/nuclear fraction and a sarcoplasmic fraction. Characterization of fractions included assaying each fraction for DNA as described previously. While the sarcoplasmic fraction had virtually no DNA, the presumptive nuclear fraction remained devoid of DNA also; most of the DNA resided in the myofibrillar fraction. Initially, further purification of the fraction was not required. It was apparent through comparison of immunoblots of the fractions, using either the anti-P-thr Ab or the anti-sarcomeric myosin Ab (NA-4), that the ~90 kDa protein was predominant in the sarcoplasmic fraction (Figure 31). The search for the identity of the ~90 kDa protein revealed several candidates at approximately 90 kDa in either the microsomal or cytoplasmic fractions.

Figure 31 Initial fractionation of C2C12 myotube cell extracts

Identification of the subcellular location of the ~90 kDa protein was accomplished through cell fractionation. C2C12 myotubes were scraped from 60 mm dishes in 200 µl NFEP buffer and Dounce homogenized. The cell suspension was centrifuged at 1,000 x g for 15 minutes forming two distinct fractions, a myofibrillar/nuclear and sarcoplasmic fraction. Fractions were loaded on an equal protein basis and separated by 8% PAGE in duplicate. Gels were transferred to a PVDF membrane and immunoblotted against either sarcomeric myosin or anti-P-threonine

Immunoblots indicate that the NA-4 Ab, bound to a single 200 kDa band in the myofibrillar nuclear fraction identifying sarcomeric myosin while no binding was observed in the sarcoplasmic fraction. Immunoblots using the anti-P-threonine Ab indicate the predominance of a single band at ~90 kDa protein in the sarcoplasmic fraction. Results indicate the separation of two distinct cellular fractions and that the ~90 kDa protein resides in the sarcoplasmic fraction of C2C12 myotubes.


myo.
nuc.
sarc.
Anti-myosin (NA4)
200 kD
Anti-P-threonine
90 kD

The search results suggested that further fractionation was required to identify which fraction, within the sarcoplasmic fraction, contained the ~90 kDa phospho-protein. Fractionation by centrifugation at 100,000 x g revealed that ~90 kDa phospho-proteins exist in both the microsomal and cytoplasmic fraction (Figure 32). The ~90 kDa phospho-protein band in the microsomal fraction declined with βAA treatment, while the ~90 kDa band of the cytoplasmic fraction increased with βAA stimulation.

Developmental Time Course

Identification of the ~90 kDa protein was further investigated through examination of ~P over a developmental time course. Cell extracts were prepared from untreated C_2C_{12} at various stages of development: from rapidly dividing myoblasts to fully differentiated myotubes. Cell extracts from those time points were then immunoblotted against the anti-P-thr Ab. The same extracts were run in parallel to NA-4 (sarcomeric myosin) for comparison to a well defined muscle-specific protein. The same cell extracts were run on an 8% PAGE and the gel stained to visualize the protein profile of the cell extracts across the development time course. Results indicate the ~90 kDa protein represents a protein which is present from early development through differentiation. Phosphorylation of the ~90 kDa protein is developmentally regulated and is further confirmed by its paralleled expression to a well defined muscle specific protein, SM (Figure 33).

Figure 32 Anti-P-threonine immunoblot against the sarcoplasmic fraction of C2C12 myotube cell extracts

The immunoblot presented represents a further fractionation of the sarcoplasmic fraction into its two constituitive fractions, the microsomal and cytoplasmic fractions. Lanes represented: (A) myofibrillar fraction, (B) sarcoplasmic fraction, (C) microsomal fraction, and (D) cytoplasmic fraction.

10 µl total protein representing each fraction was separated on 8% PAGE at 165 volts and transferred to a PVDF membrane. Immunoblotting using an anti-P-threonine Ab suggested that the ~90 kDa protein resides predominantly in the microsomal fraction.

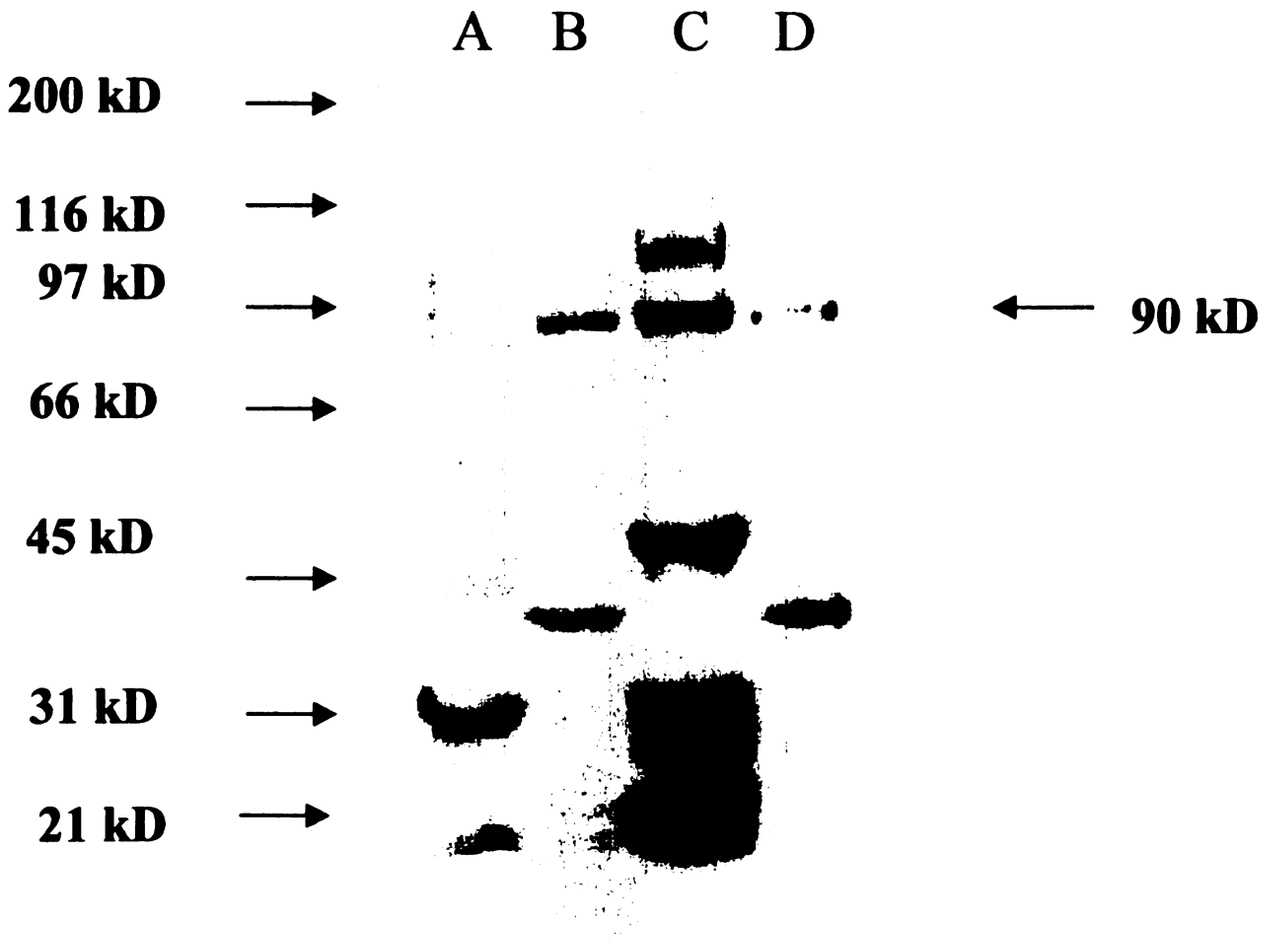

A B C D
200 kD
116 kD
97 kD
66 kD
45 kD
31 kD
21 kD
90 kD

Figure 33 Anti-P-threonine developmental time course immunoblot against C2C12 myotube cell extracts

A developmental time course study was undertaken to further identify characteristics fo the ~90 kDa protein. Cell extracts were prepared from C2C12 cells through development, from rapidly dividing myoblasts to mature myotubes. Lanes represented: (A)-(C) rapidly dividing myoblasts, (D) confluent myoblasts, (E) 24 hours post confluence, (F) 48 hours post confluence, (G) 96 hours post confluence, (H) 144 hours post confluence.

10 μg total protein from C2C12 myotube cell extracts across the development time intervals were run on 8% PAGE and transferred to PVDF membrane.

Immunoblotting was accomplished using either an anti-sarcomeric myosin Ab (NA-4) or an anti-P-threonine Ab. The profile observed at ~90 kDa in the anti-P-threonine immunoblot was compared to the NA-4 immunoblot which represents a well defined muscle specific developmentally expressed protein.

The ant-P-threonine immunoblot suggests that the phosphorylation of the ~90 kDa protein is developmentally regulated and parrallels the expression of sarcomeric myosin.

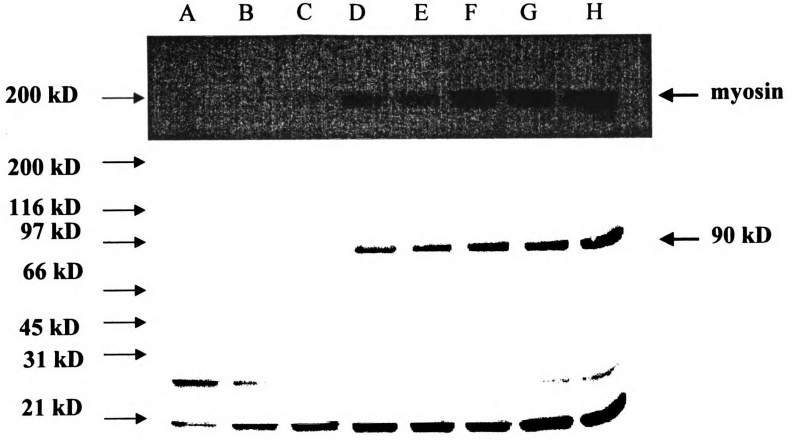

A
B
C
D
E
F
G
H
200 kD
myosin
200 kD
116 kD
97 kD
90 kD
66 kD
45 kD
31 kD
21 kD

Figure 34 Anti-P-threonine two-dimensional electrophoresis against the C2C12 myotube sarcoplasmic fraction

Further identification of the ~90 kDa protein was attempted through 2-D analysis of the sarcoplasmic fraction. 25 µl sample representing approximately 10 µg total sarcoplasmic fraction protein was separated on a 3.5% acrylamide tube gel cross-linked with piperazine diacrylamide containing 9M urea and 2% Bio-Lyte ampholytes (1 part 3/10 and 2 parts 5/7) % acrylamide tube gel. The second dimension was separated on 8% PAGE at 165 V and transferred to PVDF membrane.

Comparison of the anti-P-threonine immunoblot to 2-D standards revealed the ~90 kDa protein to migrate at a pI of approximately 5.6.

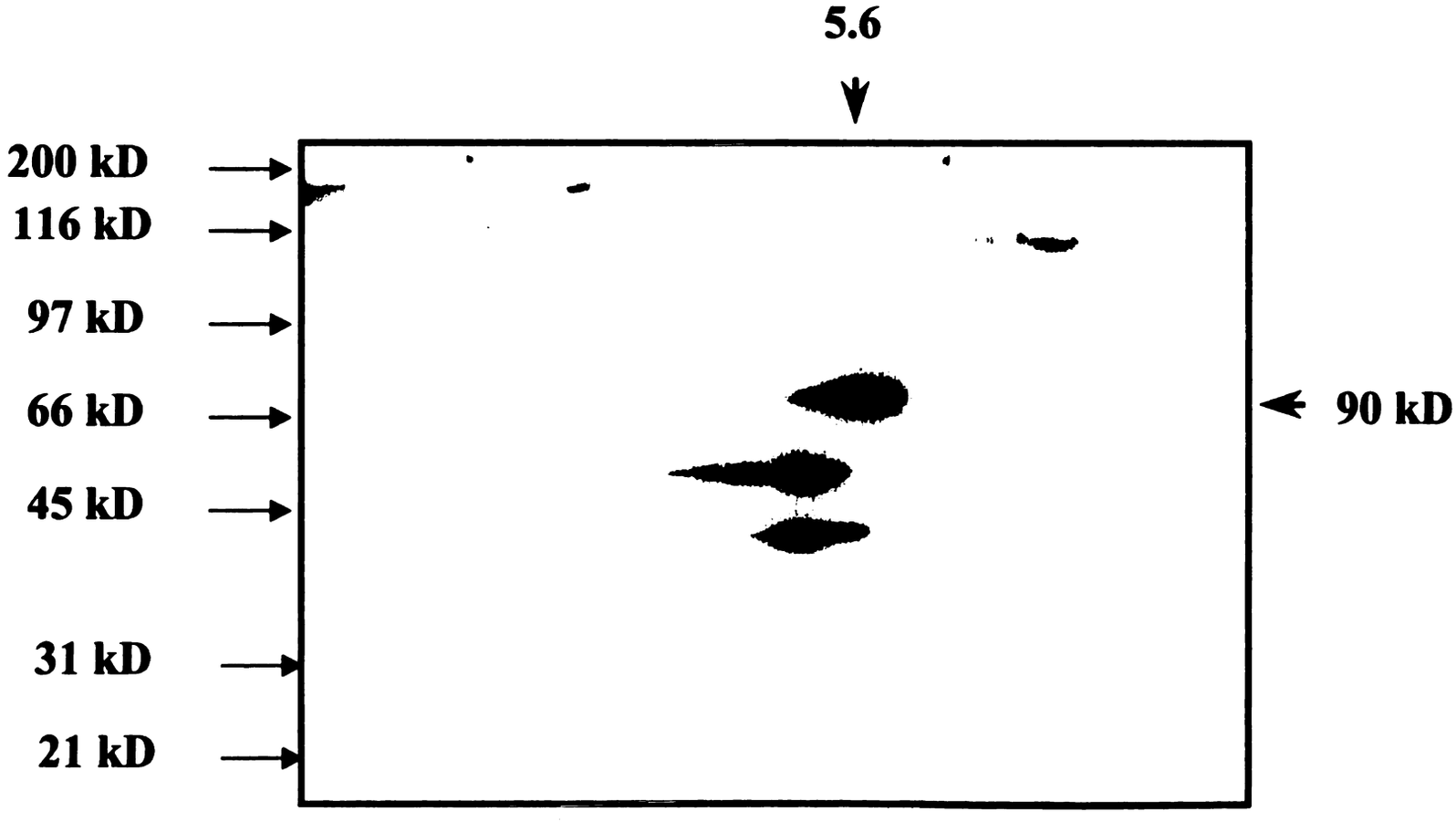
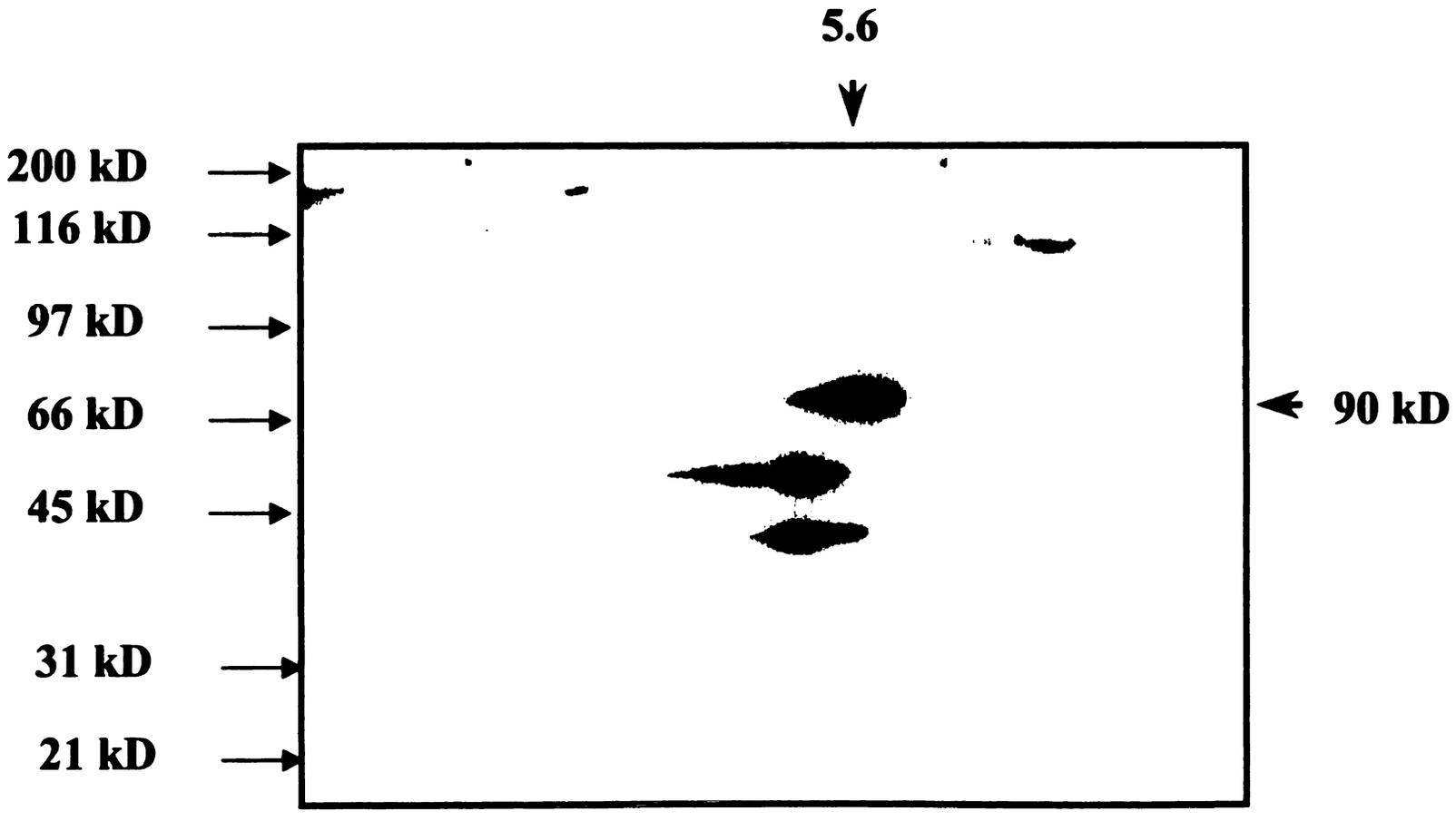

5.6
200 kD
116 kD
97 kD
66 kD
45 kD
31 kD
21 kD
90 kD

Figure 34 Anti-P-threonine two-dimensional electrophoresis against the C2C12 myotube sarcoplasmic fraction

Further identification of the ~90 kDa protein was attempted through 2-D analysis of the sarcoplasmic fraction. 25 µl sample representing approximately 10 µg total sarcoplasmic fraction protein was separated on a 3.5% acrylamide tube gel cross-linked with piperazine diacrylamide containing 9M urea and 2% Bio-Lyte ampholytes (1 part 3/10 and 2 parts 5/7) % acrylamide tube gel. The second dimension was separated on 8% PAGE at 165 V and transferred to PVDF membrane.

Comparison of the anti-P-threonine immunoblot to 2-D standards revealed the ~90 kDa protein to migrate at a pI of approximately 5.6.


5.6
200 kD
116 kD
97 kD
66 kD
45 kD
31 kD
21 kD
90 kD

Two-Dimensional (2-D) Electrophoresis

Positive identification of the protein remained elusive and more information was essential for data-base searching. Two-dimensional electrophoresis was undertaken using 20 ul representing 10 μg of the sarcoplasmic fraction to approximate the ~90 kDa protein's pI. Two-dimensional electrophoresis revealed a protein/s at ~90 kDa and by comparison to two-dimensional standards (Bio-Rad, Hecules CA) revealed the isoelectric point (pI) of the responsive protein to be approximately 5.6 (Figure 34).

Protein Purification and N-terminal Sequencing

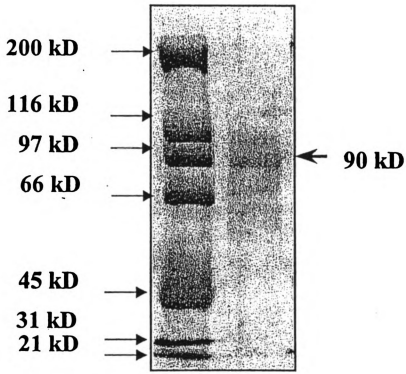
Following two-dimensional gel electrophoretic analysis and unsuccessful data-base searching, N-terminal sequencing was attempted at the Michigan State University Macromolecular Structure Facility. Sequencing required the acquisition of pure 90 kDa sample. Cell extracts were prepared from C2C12 myotubes and run on an 8% preparative gel. The ~90 kDa band of interest was dissected out of the gel and subjected to elution in 1x TBE. The slurry was then centrifuged at 3,000 x g for 30 minutes and supernatant saved for analysis. The supernatant was then dialyzed against .1x TBE for 2 hours followed by water overnight. The dialate was removed from tubing and concentrated using a Centricon device; pore size 30 kDa. The sample was run on an 8% PAGE, transferred to PVDF membrane, and stained with amido black stain; thus revealing a prominent band at ~90 kDa and two other less intense bands at ~60 and 30 kDa. The latter bands may represent degradation products of the ~90 kDa band, as it is highly improbable that these bands were excised with the ~90 kDa band initially (Figure 35).

Figure 35 Coomassie Blue stained purified ~90 kDa protein transfer

An attempt was made to purify the ~90 kDa protein from PAGE for N-terminal sequencing. Total C2C12 myotube cell extract was separated on an 8% PAGE preparative gel. The ~90 kDa band was excised and eluted from the gel. The eluted sample was concentrated, run on 8% PAGE, and transferred to a PVDF membrane. The membrane was stained with Coomassie blue stain to verify the existence of a single band at ~90 kDa.

The stained membrane indicates the presence of three light but distinct bands. The bands present are at ~90 kDa and two others at approximately 60 and 30 kDa, presumably degradation products.

The presence of the two extra bands at such a lower migration suggest that these bands may represent possible degradation products of the ~90 kDa protein rather than proteins accidentally excised with the ~90 kDa band.


200 kD
116 kD
97 kD
66 kD
45 kD
31 kD
21 kD
90 kD

Several attempts were made at sequencing the protein and were unsuccessful. Tryptic digests of the isolated 90 kDa protein were also prepared and sequence analysis attempted and failed. Potential problems may lie in a blocked N-terminus or multiple 90 kDa proteins.

Identification of a 90 kDa Protein Candidate

The search for the identification of the 90 kDa protein revealed one particularly favorable candidate: a 90 kDa muscle specific sarcoplasmic protein. The sarcoplasmic reticulum (SR) is a membranous system of tubules and cisternae (reservoirs for Ca^{++}) that forms a closely meshed network around each myofibril. A protein unique to skeletal muscle is represented by the 90 kDa junctional sarcoplasmic reticulum (JSR) protein. First identified and characterized by Guo et al. (1994), the 90 kDa protein is a membrane protein specifically localized to the terminal cisternae of the skeletal muscle SR and is a substrate for the endogenous protein kinase of the triads. Guo et al. (1994) suggest that the localization and phosphorylation of this protein may play an important role in skeletal muscle triad junction. The 90 kDa protein is especially enriched in the junctional face membrane, a membrane preparation enriched with junctional specific proteins such as the ryanodine receptor/Ca^{++}-release channel and triadin (Knudson, 1993 and Campbell, 1987), and is believed to be an integral membrane protein of the junctional face membrane (Guo et al., 1994). The 90 kDa protein is a substrate for the endogenous protein kinase present in skeletal muscle triads. It is known that isolated skeletal muscle triads contain kinase systems that specifically ~P the junctional proteins. Imagawa et al. (1987) report that

isolated triads contain two kinds of endogenous protein kinases. One is a Ca^{++}/calmodulin dependent protein kinase and the other is neither dependent on Ca^{++}/calmodulin nor dependent on cAMP; this kinase was termed the intrinsic protein kinase. The function of the phosphorylation system is presently unknown; however, the selective association of the intrinsic protein kinase with the junctional membrane suggests that it is involved in regulation of the function of this morphologically specialized structure the JSR (Imagawa et al., 1987, Guo et al., 1994). The phosphorylation of the 90 kDa protein indicates that the protein has a functional domain in the junctional region, where excitation-contraction coupling occurs (Imagawa et al., 1987, Guo et al., 1994). A distinguishing characteristic of the 90 kDa protein is that the migration of the 90 kDa protein is affected by sulphydryl reagents. Under reducing conditions, it migrates as a single band at 90 kDa. In the presence of N-ethylmaleimide (NEM), a major band of 170 kDa appears in addition to the 90 kDa band, the molecular mass of which indicates that it may be composed of dimers of the 90 kDa protein. The physiological consequence and function of the dimeric complex are currently unknown.

Immunocytochemical labeling indicates the predominance of the 90 kDa protein in the type II (fast twitch) fibers of skeletal muscle and that the staining pattern corresponds to the interphase between the A and I band suggesting that the 90 kDa protein is a JSR protein confined to the triad region. The pattern of staining is similar to those of dihydropiridine receptor, calsequestrin, and triadin. This is probably due to the greater abundance of t-tubules and JSR in type II fibers.

Immunoprecipitation of a 90 kDa Protein

Cell extracts were prepared from either control myotubes or myotubes treated 4 hours with isoproterenol. Initial separation of the myofibrillar/nuclear and sarcoplasmic fraction were prepared as described earlier. Following the final centrifugation of the sarcoplasmic fraction to microsomal fraction, the pellet was resuspended in 200 ul resuspension buffer (RS) buffer (50 mM Tris-HCl (pH 6.2), 150 mM NaCl, 1% Triton-X-100, .1% SDS, 20 mM NaF, 100 kalikrien inactivating units/ml aprotinin-Sigma Chemical Co., St. Louis, MO) at 4° C for 1 hour. The sample was centrifuged at 100,000 x g for 30 minutes to remove any insoluble fractions. A 100 µl aliquot of the supernatant was taken for analysis by Western blot. The remaining 100 µl of supernatant was incubated with 100 ul RS buffer + BSA (1 mg/ml) and 40 µl of the mouse monoclonal antibody VF1C to the 90 kDa junctional sarcoplasmic reticulum protein (Affinity Bioreagents, Golden, CO) for 1 hour with gentle mixing. At 1 hour, 40 µl of goat-anti-mouse Sepharose-G (#62-6541) (Zymed Laboratories, San Francisco, CA) slurry (1:1, Sepharose-G:RS buffer + BSA at 1 mg/ml) was added to the initial sample/Ab solution and further incubated at 4° C for 4 additional hours with gentle mixing. Samples were centrifuged, at maximum setting, in a microfuge for 5 seconds and the pellet washed a total of 3 times in wash buffer (100 mM Tris-HCl (pH 7.4), .2 M NaCl, 20 mM NaF). Following the final wash, the pellet was resuspended in 50 µl 1x treatment buffer. An aliquot was removed and mixed 1:1 with 2x treatment buffer + MCE, boiled for 1 minute and run on SDS-PAGE as described previously.

Immunoprecipitation of the 90 kDa protein with the VFC1 antibody (Affininty Bioreagents, Golden, CO) reveals that the 90 kDa JSR protein is not the βAA responsive protein identified in C2C12 myotubes (Figure 36). Immunopreciptiation as described previously did reveal the 90 kDa JSR protein in the microsomal fraction of the fractionated cell extracts (Figure 37). A parallel immunoblot using the anti-P-thr Ab did not reveal a phosphorylated 90 kDa protein in the immunoprecipitate, indicating that the immunoprecipitated protein was not the βAA responsive protein (Figure36). Cytoplasmic fractions, however, did reveal a βAA responsive phosphorylated ~90 kDa protein (Figure 36). The anti-P-thr immunoblot presents an increased phosphorylation response to βAA stimulation in comparison to control fractionated cell extracts representing the cytoplasmic fraction. The βAA-responsive ~90 kDa protein in C2C12 myotubes is a cytoplasmic protein. The supernatant from the immunoprecipitation, which would represent the microsomal fraction, was also run on an 8% SDS-PAGE and immunoblotted using the anti-P-thr antibody. The anti-P-thr immunoblot of the microsomal fraction of control and ISO stimulated myotubes revealed a ~90 kDa protein which appears to respond in an opposite manner to that of the up-regulated βAA responsive ~90 kDa protein in the cytoplasmic fraction (Figure 36). In regard to the initial separation of the sarcoplasmic fraction into two constituent fractions; it is now apparent that the immunoblot was mistakingly interpreted. The predominance of several proteins in the lower ranges (MW 30-40 kDa) not present in the initial total sarcoplasmic fraction. The intensity of the lower bands (MW 30-40 kDa) indicates that the microsomal fraction was much more concentrated than originally interpreted. As a result, the

Figure 36 Identification of the subcellular location of an ~90 kDa protein in C2C12 myotubes

Following an extensive search for the identification of the ~90 kDa protein; the existence of several candidates in either the microsomal or cytoplasmic led to the "best-candidate" immunoprecipitate. C2C12 myotubes were stimulated with isoproterenol (10^{-5} M) for 4 hours and fully fractionated to the microsomal and cytoplasmic fractions.

The microsomal fraction of the 2% FBS control and isoproterenol stimulated cells were immunoprecipitated with an anti-90 kDa junctional sarcoplasmic reticulum Ab, separated on 8% PAGE, transferred to a PVDF membrane, and immunoblotted with either the anti-90 kDa junctional sarcoplasmic reticulum Ab or the anti-P-threonine Ab.

Pictured initially, is the original fractionation of the sarcoplasmic fraction (S) to the component micosomal (M) and cytoplasmic fractions (C) for reference. The second panel represents the anti-P-threonine Ab immunoblot against the control immunoprecipitate (CI) and the isoproterenol stimulated immunoprecipitate (TI). It is apparent that no activity is present in the anti-90 kDa immunoprecipitate anti-P-threonine immunoblot. This observation indicates that the 90 kDa junctional sarcoplasmic protein is not the elusive ~90 kDa protein.

The third panel from the left represents the microsomal fraction of both the 2% FBS control (CM) and isoproterenol stimulated (TM) myotube cell extracts. The anti-P-threonine immunoblot reveals that at an ~90 kDa protein is present in both extracts, however; the response to isoproterenol stimulation is less intense than the 2% FBS control. The ~90 kDa beta-adrenergic responsive protein can not be in the microsomal fraction.

The final panel represents anti-P-threonine immunoblot of the 2% FBS control (CC) and isoproterenol stimulated (TC) cytoplasmic myotube cell fractions. A pronounced response is observed at ~90 kDa in the cytoplasmic fraction of isoproterenol stimulated myotubes. The ~90 kDa protein resides in the cytoplasmic sub-cellular fraction of C2C12 myotubes.

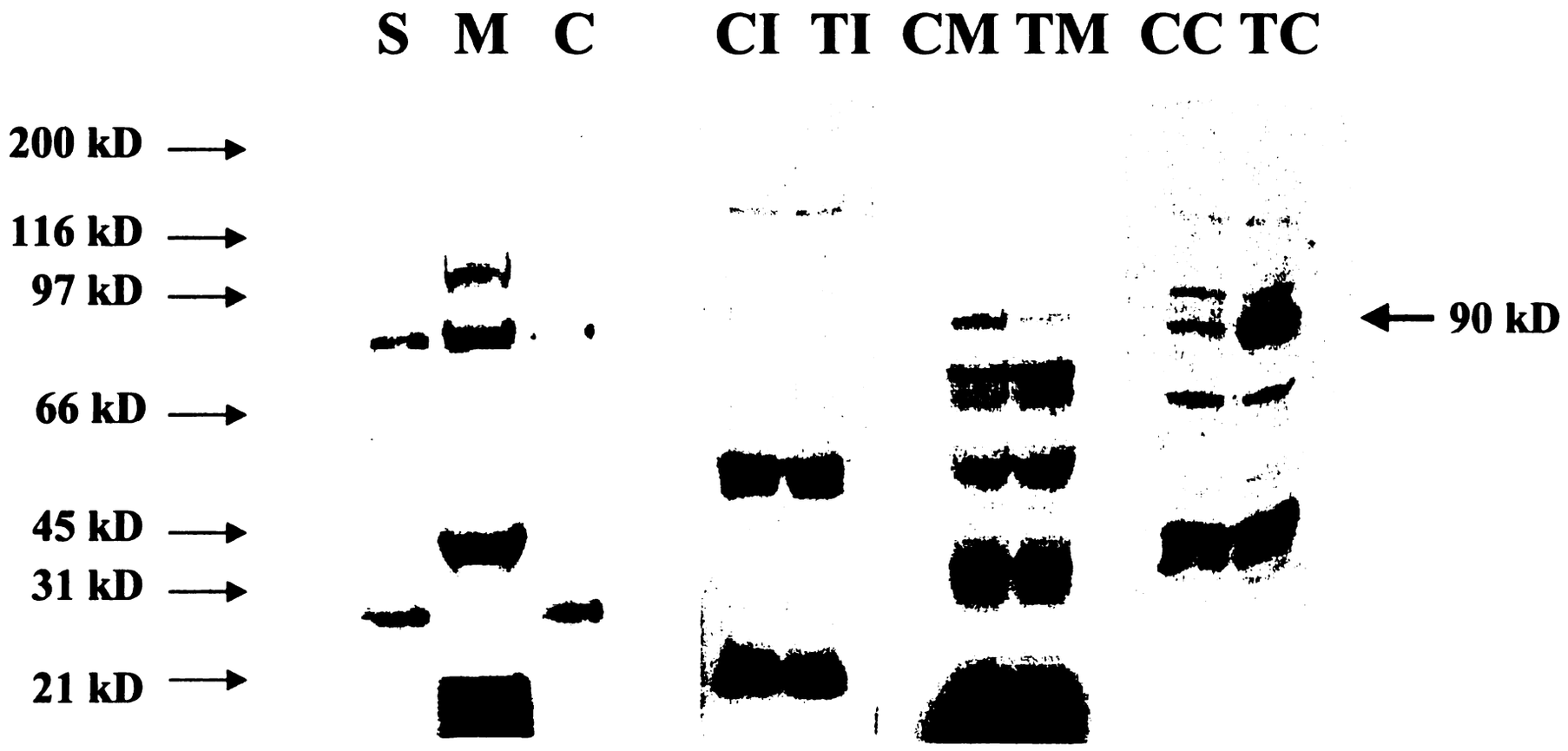
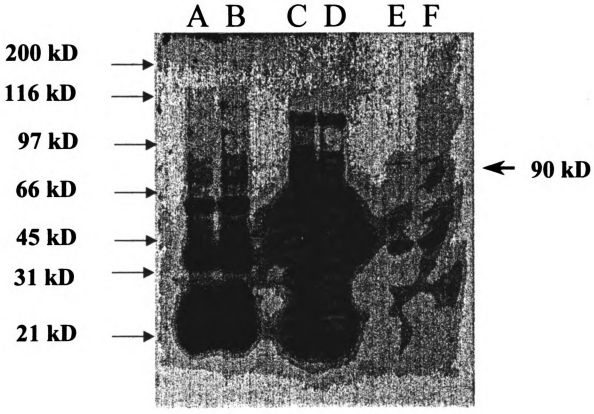

S M C
CI TI CM TM CC TC
200 kD
116 kD
97 kD
66 kD
45 kD
31 kD
21 kD
90 kD

Figure 37 VF1-C immunoblot against immunoprecipitated ~90 kDa protein of isoproterenol stimulated and control C2C12 myotube microsomal fractionated cell extracts

The blot presented represents the VF1-C immunoblot against immunoprecipitated 90 kDa junctional sarcoplasmic reticulum protein from 2% FBS control or isoproterenol stimulated C2C12 microsomal myotube cell fractions. Lanes represented: (A) total sarcoplasmic fraction of 2% FBS control myotubes, (B) total sarcoplasmic fraction of isoproterenol stimulated myotubes, (C) immunoprecipitated 2% FBS control microsomal fraction, (D) immunoprecipitated isoproterenol stimulated microsomal fraction, (E) cytoplasmic fraction of 2% FBS control myotubes, (F) cytoplasmic fraction of isoproterenol stimulated myotubes.

Data presented in the immunoblot indicate that the VF1-C immunoprecipitation worked. The 90 kDa band resides in the microsomal fraction where it is reported to be predominant. In addition, the immunoblot does not suggest any stimulation in the amount of 90 kDa junctional sarcoplasmic reticulum protein further supporting the notion that the ~90 kDa beta-adrenergic stimulated protein is not the 90 kDa junctional sarcoplasmic reticulum protein.


A B C D E F
200 kD
116 kD
97 kD
66 kD
45 kD
31 kD
21 kD
90 kD

intensity of a band at ~90 kDa was misinterpreted. In reality, the ~90 kDa band in the cytoplasmic fraction was as intense as the ~90 kDa band in the initial sarcoplasmic fraction. The observation that the ~90 kDa protein was as intense in the, diluted, cytoplasmic fraction should have suggested the predominance of the protein in that fraction. The fractionation data indicate not only that several ~90 kDa proteins may be present in the sarcoplasmic fraction but that the βAA stimulated phosphorylation response may have been less obvious in earlier non-fractionated samples due to the apparent decrease in phosphorylation of the ~90 kDa protein of the microsomal fraction. The presence of multiple ~90 kDa proteins may also explain the difficulties encountered upon purification of the ~90 kDa band from cell extract preparative gels used for initial sequencing procedures where sequence analysis appeared indeterminable. The identity of the ~90 kDa cytoplasmic protein remains undetermined.

Another candidate for the ~90 kDa βAA responsive cytoplasmic protein may be phosphorylase. Phosphorylase represents a cyotplasmic protein which is ~P in response to adrenergic stimulation and appears to migrate at approximately at the same MW on an acrylamide gel (Alvarez et al., 1992). The only problem lies in the fact that the ~P site on phosphorylase is at a serine residue (Rawn, 1989). I am using an anti-P-thr Ab. Zymed laboratories (San Francisco, CA) assures that the anti-P-thr Ab does not cross-react with ser~P or tyr~P. It may be that the Ab is cross-reacting with the ser~P or perhaps with the pyridoxal-phosphate present in phosphorylase. The same cell extracts will have to be immunoprecipitated with an anti-phosphorylase Ab and examined as were the previous

JSR 90 kDa protein immunoprecipitates to be certain if the phosphorylated protein is glycogen phosphorylase.

βAA stimulation of C2C12 myotubes increaes the ~P of an ~90 kDa cytoplasmic protein. Stimulation of various βAA signal transduction pathway components results in a similar increase to that observed upon stimulation at the βAR (Figure 38). The identity of the ~90 kDa cytoplasmic protein and its role in βAA-induced events in skeletal muscle remain undetermined.

Figure 38 Summary figure- Chapter 3

The diagram represents the overall possibilities in "cross-talk" among signaling pathways. In review: stimulation at the β-adrenergic receptor by isoproterenol (10^{-5} M) resulted in increased phosphorylation of an ~90 kDa cytoplasmic protein in C2C12 myotubes relative to the 2% FBS control. Furthermore, modulation of various β-adrenergic pathway components supported the observation that the increased phosphorylation was occurring through a cAMP dependent protein kinase pathway. Stimulation at the β-adrenergic receptor by either isoproterenol (10^{-5} M) or ractopamine (10^{-6} M) resulted in approximately threefold increase in phosphorylation response at the ~90 kDa protein. The addition of either cAMP (1 mM), forskolin (10^{-4} M), or cholera toxin (500 ng/ml) also resutled in a similar increase in phosphorylation relative to the 2% FBS control. The results indicate that the increased phosphorylation of a ctyoplasmic ~90 kDa protein observed upon β–adrenergic stimulation of C2C12 myotubes is occurring through a cAMP dependent protein kinase pathway. The identity of the ~90 kDa cytoplasmic protein remains undetermined.

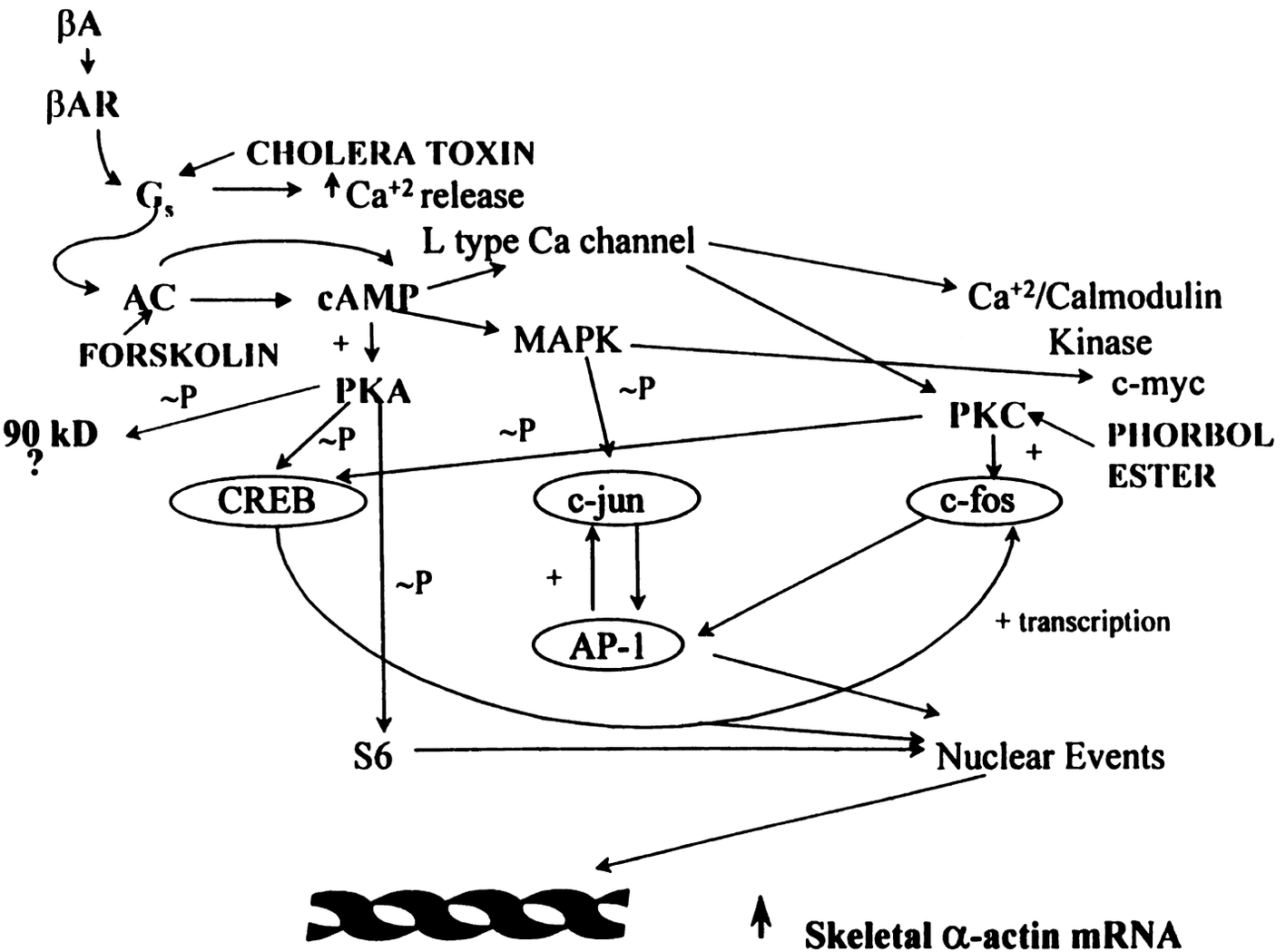

βA
βAR
CHOLERA TOXIN
G_s
↑Ca^{+2} release
L type Ca channel
Ca^{+2}/Calmodulin
Kinase
AC
cAMP
MAPK
c-myc
FORSKOLIN
+
~P
PKA
~P
PKC
PHORBOL
ESTER
90 kD
?
~P
~P
+
CREB
c-jun
c-fos
~P
+
+ transcription
AP-1
S6
Nuclear Events
Skeletal α-actin mRNA

Figure 38 Summary figure- Chapter 3

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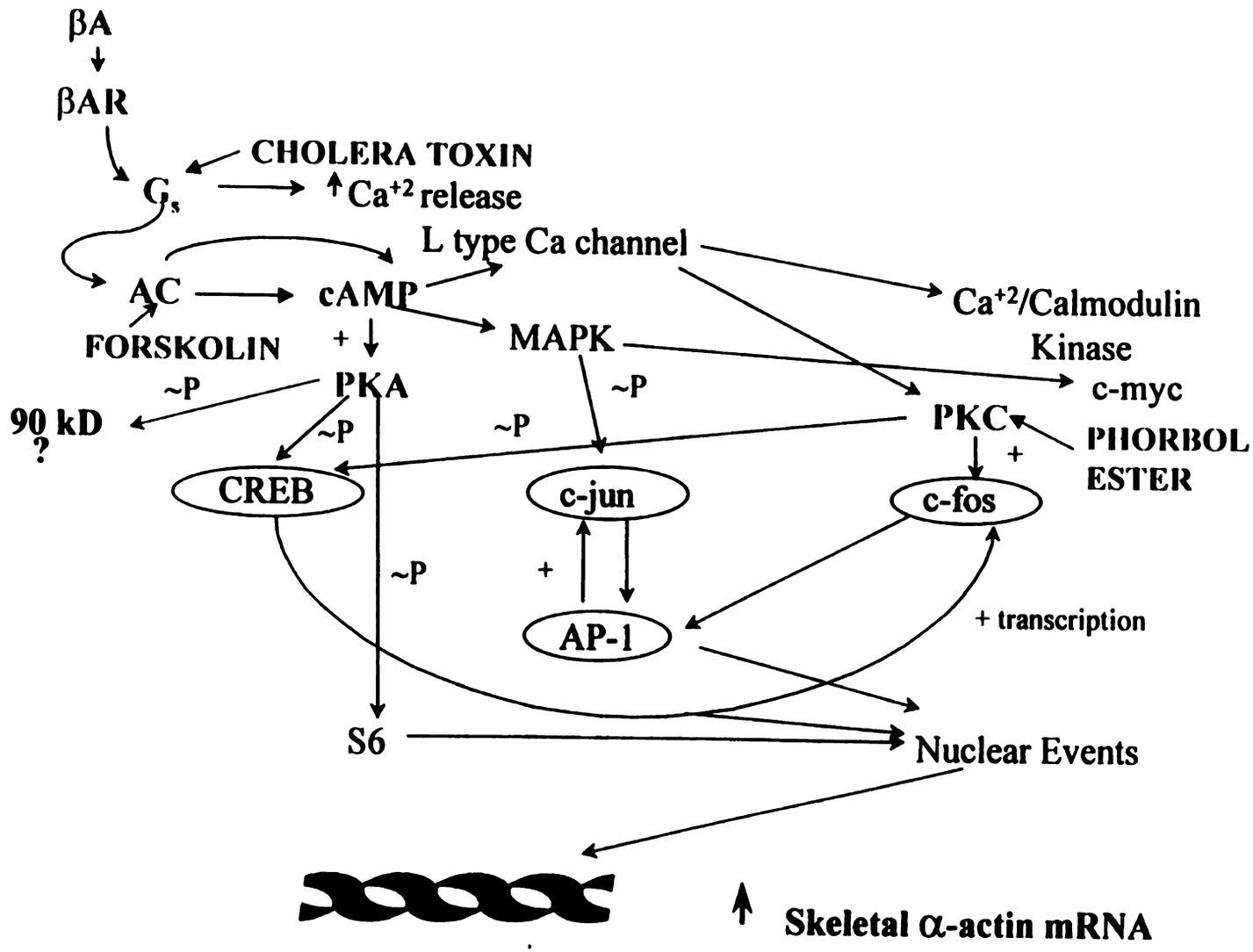

βA
βAR
CHOLERA TOXIN
G_s
↑Ca^{+2} release
L type Ca channel
AC
cAMP
Ca^{+2}/Calmodulin Kinase
FORSKOLIN
+
MAPK
c-myc
~P
PKA
~P
90 kD ?
~P
PKC
PHORBOL ESTER
+
CREB
c-jun
c-fos
~P
+
AP-1
+ transcription
S6
Nuclear Events
Skeletal α-actin mRNA

CONCLUSION

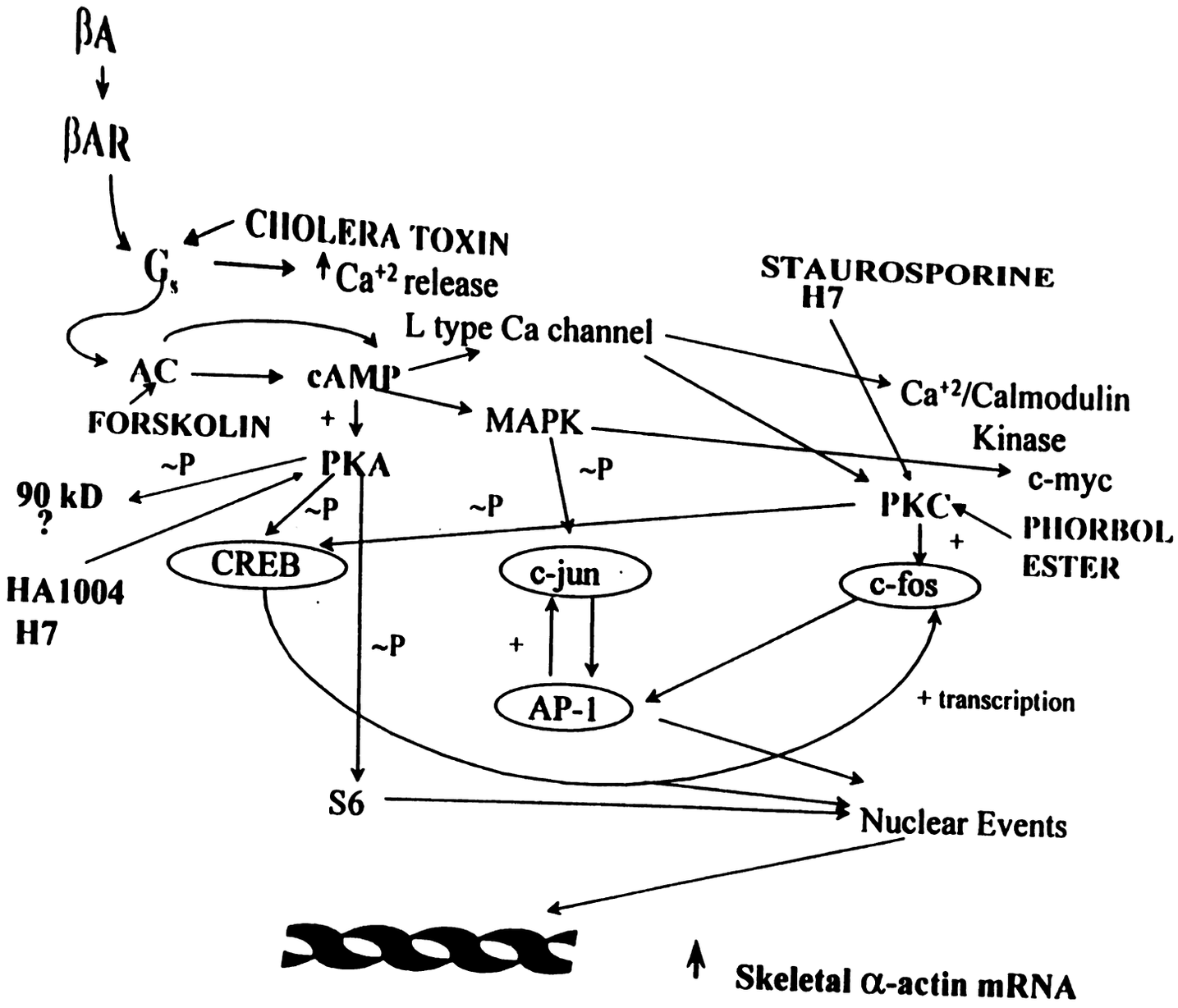
The data presented indicate that βAA stimulation of C2C12 myotubes increases mRNA abundance for SKA and increases the phosphorylation of an ~90 kDa cytoplasmic protein. The pathway modulators and results are depicted in the summary figure (Figure 39). The significance lies in the observation that βAA stimulation of muscle-specific gene expression and ~P in the immortalized skeletal muscle cell line, C2C12, are occurring through a cAMP-dependent cascade mechansim. Interpretation of data in the literature suggests an inconsistent response to βAA stimulation. Inconsistencies may be interpreted as inherent differences in regard to species and tissue type expression of βAR subtypes and differing pharmacological properties at receptors of various agonists. Data presented support βAA stimulation of skeletal muscle specific events through a βAR mediated cAMP-dependent cascade mechanism.

Figure 39 Dissertation summary figure

In summary; data indicate that β-adrenergic stimulation of muscle-specific events in C2C12 cells is occurring through a cAMP-dependent protein kinase cascade of events.

β-adrenergic stimulation of C2C12 myotubes increases skeletal α-actin mRNA abundance and this response can be blocked by the addition of propranolol or HA1004. The lack of a response upon the addition of β-adrenergic receptor antagonist or protein kinase A inhibitor indicate that β–adrenergic stimulation of skeletal α-actin mRNA abundance is occurring through a cAMP-dependent protein kinase cascade.

Furthermore, β-adrenergic stimulation of C2C12 myotubes increases the phosphorylation of an ~90 kDa cytoplasmic protein. The increased phosphorylation at ~90 kDa was also observed through stimulation of various β-adrenergic pathway components. The data indicate that the increased phosphorylation of the ~90 kDa protein is occurring through a cAMP-dependent protein kinase pathway. The identity of the ~90 kDa protein remains undetermined.


βA
βAR
CHOLERA TOXIN
G_s
↑Ca^{+2} release
STAUROSPORINE
H7
L type Ca channel
AC
cAMP
Ca^{+2}/Calmodulin
Kinase
FORSKOLIN
+
MAPK
c-myc
~P
PKA
90 kD
?
~P
~P
~P
PKC
PHORBOL
ESTER
+
CREB
c-jun
c-fos
HA1004
H7
~P
+
AP-1
+ transcription
S6
Nuclear Events
Skeletal α-actin mRNA

IMPLICATIONS

The magnitude of response relative to altered body composition of livestock species treated with a βAA is dramatic and reproducible across species (Muir, 1988). To date, βAA are not federally approved for use as a growth promotants/metabolism modifiers in livestock species. Government approval of βAA usage is centered on the issue of βAA residues in meat. The fact that βAA are not approved for use suggests that further investigation into their mechanism of action is required in order to optimize this particular strategy. Further complications relative to βAA usage include the issue of meat tenderness.

Considering the dramatic effects on body composition, the use of βAA in a livestock production setting merits strong attention. A major focus in United States meat-animal production settings has been the meat tenderness issue. Unfortunately, use of βAA to has been shown to not only increase lean tissue gain, but decrease meat tenderness across species (Wheeler et al., 1992, Pringle et al., 1993, Gwartney et al., 1991).

Research regarding the correlation between βAA treatment and meat tenderness has received considerable attention. βAA treatment has been reported to increase mRNA

abundance for the protease inhibitor calpastatin (Koohmaraie et al., 1991). Furthermore, it has recently been reported that the calpastatin promoter region contains domains responsive to cAMP-dependent protein kinase (PKA) stimulation (Cong et al., 1998).

These findings indicate a direct relationship between βAR stimulation and decreased meat tenderness. These findings are not very promising for strong advocates of βAA's. If the product is unacceptable to the consumer there is no benefit to the producer regardless of the advantages in muscle deposition and fat reduction.

Further investigation of the βAA signal transduction pathway is essential if that production strategy is to be utilized. An investigation into the interaction across signaling pathways as expressed earlier may address previous questions or expose new options to modify the βAA response to optimize product approval by the government and the consumer.

In relation to further examination of the mechanism of action of βAA; it may very well be that one particular strategy may not be sufficient to meet producer and consumer demands. Information regarding mechanism of action may indicate a synergy between growth promotants or related strategies. Furthermore, genetic manipulation of livestock species can not be left unmentioned. A strategy which optimizes muscle growth and fat reduction yet also meets producer/consumer demands may be represented by a

commercially available transgenic animal fed a βAA acting in synergy to create the desired product.

APPENDICES

Appendix A

Appendix A

Polymerase chain reaction cycle set-up for the 105 bp skeletal α-actin digoxygenin labeled probe

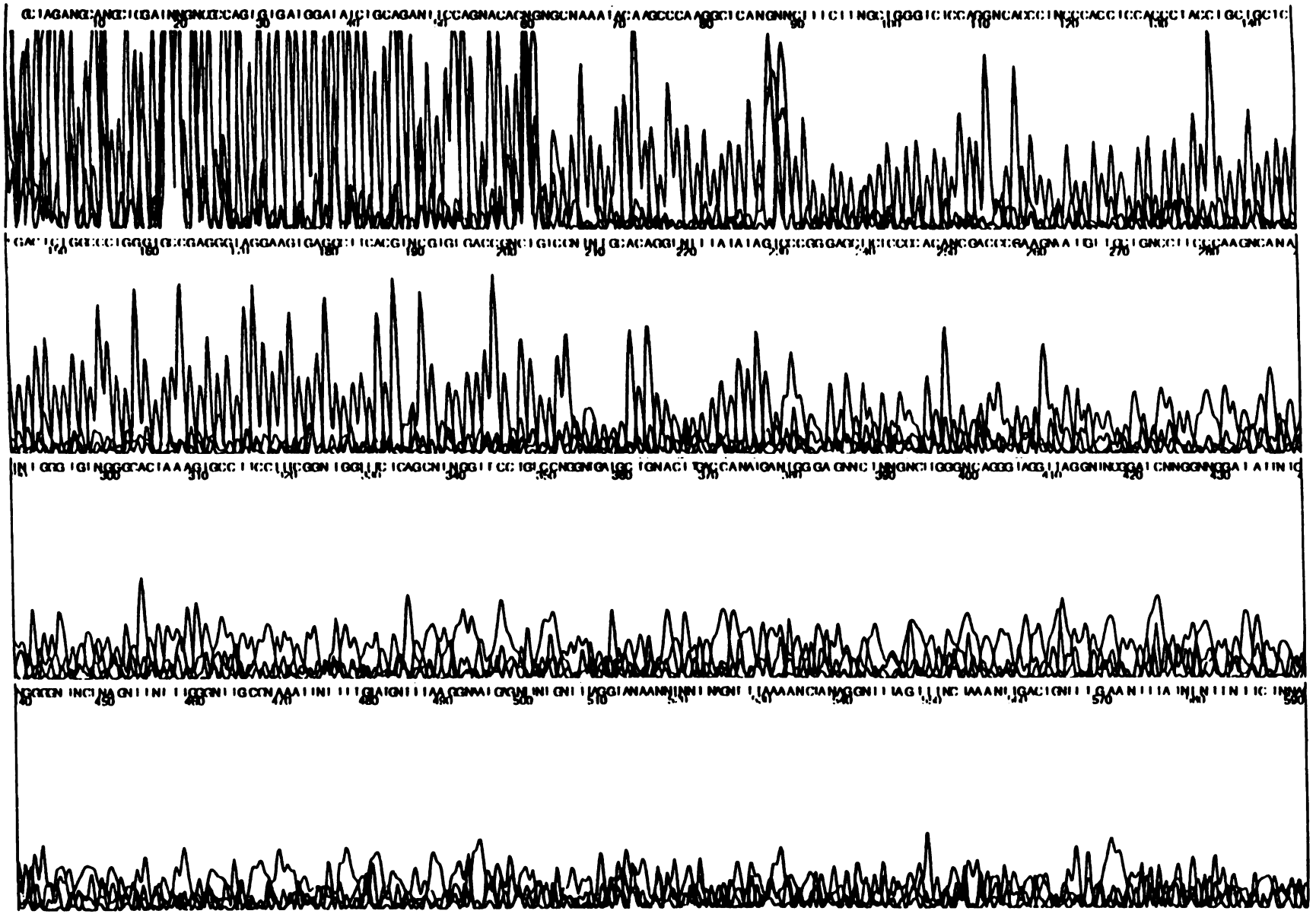
Annealing temperature formula:
4 (#GC) + 2 (#AT) = +/- 5°

°C	time	
94°C	4 minutes	
94	1 minute, 10 seconds	*3 cycles*
60	" "	
72	" "	
94	1 minute, 10 seconds	*2 cycles*
58	" "	
72	" "	
94	1 minute, 10 seconds	*2 cycles*
55	" "	
72	" "	
94	1 minute, 10 seconds	*30 cycles*
50	" "	
72	" "	
4	hold	

Appendix B

Appendix B

Dyedeoxy chromatogram of the Bam HI/Pst-I 931 bp fragment



Appendix C

Appendix C

Dyedeoxy chromatogram of the Bam HI/Pst-I 451 bp fragment

Appendix D

Appendix D

Polymerase chain reaction cycle set-up for the 546 bp 18S rRNA digoxygenin labeled probe

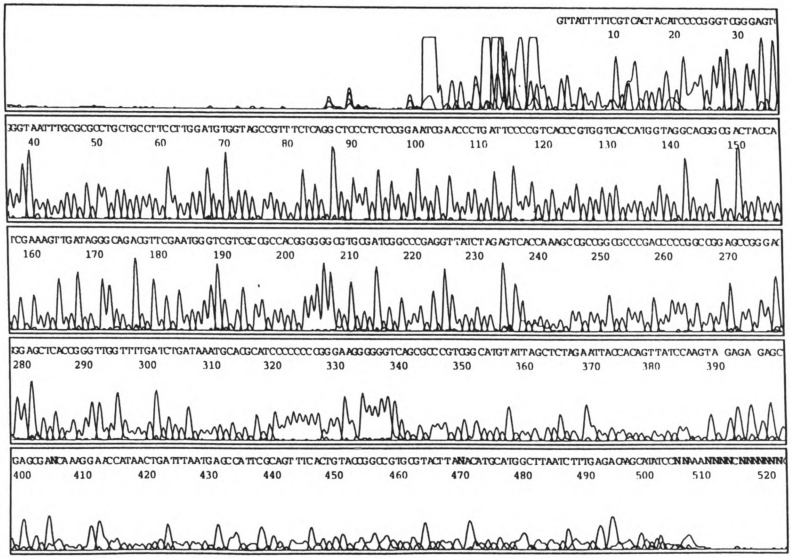
Annealing temperature formula:
4 (#GC) + 2 (#AT) = +/- 5°

°C	time	
94°C	4 minutes	
94	1 minute, 10 seconds	*3 cycles*
55	" "	
72	" "	
94	1 minute, 10 seconds	*2 cycles*
54	" "	
72	" "	
94	1 minute, 10 seconds	*2 cycles*
53	" "	
72	" "	
94	1 minute, 10 seconds	*30 cycles*
50	" "	
72	" "	
4	hold	

Appendix E

Appendix E

Dyedeoxy chromatograms of the 18S rRNA 546 bp fragment


GTTATTTTCGTCACTACATCCCCGGGTCGGGAGT
10 20 30
XGGTAATTGCGCGCTGCTGCCTTCCTTGGATGTGGTAGCCGTTTCTCAGGCTCCCTCTCCGGAATCGAACCCTGATTCCCCGTCACCCGTGGTCACCATGGTAGGCACGGCGACTACCA
40 50 60 70 80 90 100 110 120 130 140 150
TCGAAAGTTGATAGGGCAGACGTTCGAATGGGTCGTCGCCGCCACGGGGGGCGTGCGATCGCCCGAGGTTATCTAGAGTCACCAAAGCCGCCGGCGCCCGACCCCGCCGGCGGCCGGAGCCGGGAC
160 170 180 190 200 210 220 230 240 250 260 270
GGAGCTCACGGGTTGGTTTTGATCTGATAAATGCACGCATCCCCCCCCCGGGAAGGGGGGTCAGCGCCCGTCGGCATGTATTAGCTCTAGAATTACCACAGTTATCCAAGTAGAGAGAGC
280 290 300 310 320 330 340 350 360 370 380 390
GAGCGAACAAGGAACCATAACTGATTTAATGAGCCATTCGCAGTTCCACTGTACCGGCGTGCGTACTTANACATGCATGGCTTAATCTTTGAGACAGCKMTCCNNAAANNNNCNNNNNNN
400 410 420 430 440 450 460 470 480 490 500 510 520

Appendix F

Appendix F
Product index

DESCRIPTION	COMPANY	CATALOG #
Antibodies:		
Anti-dig Fab fragments	BMB	1-093-274
Anti-P-ser.	Zymed	61-8100
Anti-P-thr.	Zymed	71-8200
Anti-Mouse IgG (Alkaline phosphatase conjugate)	Sigma	A-3562
Anti-Rabbit IgG (Alkaline phosphatase conjugate)	Sigma	A-3687
Anti-Mouse IgG (Peroxidase conjugate)	Sigma	A-8924
Anti-Rabbit IgG (Peroxidase conjugate)	Sigma	A-9169
Compounds:		
cAMP	Sigma	D-0627
Cholera toxin	Sigma	C-8052
Cyclohexamide	Sigma	C-7698
Forskolin	Sigma	F-6886
HA1004	ICN	158926
H7	Sigma	I-7016
Isoproterenol	Sigma	I-6504
Phorbol ester	Sigma	P-8139
Phosphdiesterase inhibitor	Sigma	B-8279
Propranolol	Sigma	P-0884
Staurosporine	Sigma	S-4400
Kits:		
CDP-Star	BMB	0-685-627
MCK kit	Sigma	47-20
PCR probe labeling kit	BMB	1-636-090

Super-Signal Substrate for Western Blotting	Pierce	340-80
Miscellaneous:		
DEPC	Sigma	D-5758
DMEM	GIBCO	12100-46
FBS	Sigma	F-2442
TRLzol	GIBCO	10296-010

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