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Radioligand Binding of [^{3}H]CGP12177 and Efficacies of Isoproterenol, Ractopamine, and Clenbuterol on the Ligand-Regulated βAR-Adenylyl Cyclase Systems in Porcine Satellite and C2C12 Cells.

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RADIOLIGAND BINDING OF [^{3}H]CGP12177 AND EFFICACIES OF ISOPROTERENOL, RACTOPAMINE, AND CLENBUTEROL ON THE LIGAND-REGULATED βAR-ADENYLYL CYCLASE SYSTEMS IN PORCINE SATELLITE AND C2C12 CELLS

By

Ernest Benjamin Izevbigie

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ABSTRACT

RADIOLIGAND BINDING OF [^{3}H]CGP12177 AND EFFICACIES OF ISOPROTERENOL, RACTOPAMINE, AND CLENBUTEROL ON THE LIGAND-REGULATED βAR-ADENYLYL CYCLASE SYSTEMS IN PORCINE SATELLITE AND C2C12 CELLS

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Ernest Benjamin Izevbigie

The mechanism of action of beta-adrenergic agonists (βAA) on adipocytes is believed to be beta-adrenergic receptors (βAR)-mediated. Contrary to adipocytes, the mode of action of βAA on myocytes is unclear. The lack of understanding of βAA actions on myocytes is due, at least in part, to few myocyte models available for biochemical studies. Therefore, the present studies sought to provide more insights to better understand the mode of action of βAA on myocytes.

Differentiated C2C12 cell membrane preparations were incubated with increasing concentrations of [^{3}H]CGP12177 (specific activity 42.5 Ci/mmole) in the presence or absence of a 1000-fold concentration of unlabeled CGP12177 to investigate if C2C12 cells could be used as a model to study the mode of action of βAA on muscle. The specific binding of [^{3}H]CGP12177 to C2C12 cell membrane preparations was highly specific, saturable, reversible, and of high affinity (K_D = 0.2 nM). The binding maximum (B_{max}) value was calculated to be 150 fmole/mg protein, which is similar to the B_{max} value previously reported (Coutinho et al., 1990; Mersmann & McNeal, 1992) for pig adipose membrane preparations. The 10% non-specific binding of [^{3}H]CGP12177 to C2C12 cell membrane preparations reported is typical for this ligand (Lacasa et al, 1986; Mersmann & McNeal, 1992).

M2 porcine satellite cells were characterized to investigate the presence of functional βAR in these cells. Non-differentiated porcine satellite cell membrane preparations produced a linear response, but unsaturable [^{3}H]CGP12177 specific binding was observed regardless of the membrane protein concentration used (20-50 μg/ml) in non-differentiated porcine satellite cells. Little or no βAR presence was observed in differentiated cells. The radioligand studies established the presence of βAR in differentiated C2C12 cells and non-differentiated porcine satellite cells. These findings were further strengthened by the βAA-induced cAMP release.

Results indicated that all concentrations of βAA (10^{-9} to 10^{-5}) resulted in extracellular cAMP release higher than the negative control. 10^{-5} M ISO, RAC, and CLEN increased extracellular cAMP release ($P < .01$) on per unit of cellular protein. Forskolin-stimulated extracellular cAMP release was 17-fold that of the negative control.

In differentiated and non-differentiated porcine satellite cells, forskolin significantly increased extracellular cAMP release ($P < .001$) compared to the negative controls in all experiments. 10^{-5} M ISO significantly increased extracellular cAMP release ($P < .001$) in differentiated but not in non-differentiated porcine satellite cells. βAR transcripts could not be detected by RT-PCR to explain this difference.

This dissertation entitled "Radioligand binding of [^{3}H]CGP12177 and efficacies of isoproterenol, ractopamine, and clenbuterol on the ligand-regulated βAR-adenylyl cyclase systems in porcine satellite and C2C12 cells" is dedicated to my son, Ernest Osahon Izevbigie, Jr.

My son, keep your father's command, and do not forsake the law of your mother. (Proverbs 6:20)

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TABLE OF CONTENTS

TABLE OF CONTENTS (cont'd.)

TABLE OF CONTENTS (cont'd.)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (cont'd.)

CHAPTER I: INTRODUCTION

In North America, a high percentage of human dietary protein, vitamin, and mineral requirements are met through the consumption of meat and meat products. The United States Department of Agriculture (USDA) prime or high choice quality grade beef (prime refers to most marbled/fat-containing cut) was once preferred and demanded, even at higher price, by consumers. However, during the last 25 years, and due to increased public awareness of the health risks associated with chronic over-consumption of dietary energy, modern consumers prefer leaner meat products. Excess fat deposition by meat-producing animals is an economic waste to both producers and consumers. It has been estimated that it costs the meat animal production industry about $4 billion annually to deposit fat on animals and remove the fat from meat products (reviewed by Bergen & Merkel, 1991). The meat industry was not able to quickly modify their conventional production practices to meet consumer demand for leaner and less fat products and consequently consumption of red meat products declined for the past 10 years. Despite this decline, the obesity rate in the U.S. has not declined, which seems to suggest that consumption of red meat may not be the sole cause of human obesity.

Unquestionably, excess fat deposition in meat-producing animals is a problem and must be controlled. The industry has responded to this preference for leaner and less fat meat products by developing several strategies toward minimizing fat deposition in meat-producing animals; the most effective are the use of large frame, late maturing animals and feed intake control. These practices have resulted in improved lean:fat ratio, although these practices are very cumbersome and may not be cost-effective for U.S. producers.

As it relates to research, there is an increasing quest by animal scientists to develop strategies to mitigate fat while increasing lean tissue deposition in meat-producing animals. There are three ways by which this goal may be achieved: (a) increasing lean tissue deposition, (b) decreasing fat deposition, and (c) a combination of both. Research data generated over the past decade suggests that carcass composition may be manipulated by the use of exogenous growth promotants such as anabolic steroids, beta-adrenergic agonists (βAA), and growth hormone (GH). Anabolic steroids such as estrogen, estrogenic analogs, and trenbolone acetate, approved by the Food and Drug Administration (FDA), are used in ruminants (beef) to improve carcass quality. Furthermore, βAA may enhance lean tissue deposition (Ricks et al., 1984) while GH, approved for milk production in cows by the FDA, exerts lipolytic effects in steers (Schlegel et al., 1996). βAA and GH (pending FDA's approval) are more effective in non-ruminant animals such as pigs.

βAA are very effective in promoting lean tissue deposition (Bergen et al., 1989; Grant et al., 1993), while decreasing fat deposition (Barak et al., 1992) in pigs. Similar results have been reported in other meat-producing animal species such as steers (Ricks et al., 1984) and lambs (Baker et al., 1984). βAA appear to be less efficacious in poultry (Johnson, 1989). Recently, the βAA ractopamine (RAC) was reported to exert a stimulatory effect on satellite cell proliferation (Cook et al., 1994;

Grant et al., 1990). The proliferative activity of satellite cells is important for skeletal muscle growth (Allen et al., 1979). Meat animal-derived satellite cells are of great importance in animal agriculture; therefore it becomes imperative to study and understand the factors that regulate the activation of satellite cells from a dormant stage to proliferation, differentiation and subsequent fusion. Currently, most of what we know about the effects of βAA on satellite cells, particularly porcine-derived satellite cells, has emanated from studies that failed to investigate the presence or absence of βAR; there are no published data, at least to our knowledge, to indicate the presence of a functional βAR in any food animal satellite cells. The present studies seek to establish the involvement of yet another factor, βAA, that enhances the proliferative activity of porcine satellite cells besides basic fibroblast growth factor (bFGF), insulin-like growth factors (IGF-I and II), and platelet-derived growth factor-BB (PDGF-BB) (Doumit et al., 1993).

Administration of exogenous GH not only promotes muscle hypertrophy (Grant et al., 1991) but also markedly depresses fat deposition in pigs (Caperna et al., 1990; Verstegen et al., 1990) by decreasing lipogenesis (Harris et al., 1993; Kramer et al., 1993; Liu et al., 1994).

Taken together, a drastic reduction in fat deposition in meat-producing animals may not be accomplished by the use of any one practice, factor, or exogenous agent, but a combination of practices, factors and exogenous agents may result in the production of meat products in which fat supplies not more than 30% of their total calories. Of course, the overall goal is that these lean meat products may help in decreasing acute clinical cases of high dietary fat-related diseases and lower the health care cost burden on society.

CHAPTER II: LITERATURE REVIEW

Many traditional concepts of meat quality have assumed that intramuscular adipose tissue enhances meat palatability. The USDA prime or high choice quality grade beef (prime refers to high level of marbling/fat-containing meat cuts), once preferred by consumers even at a higher price, are no longer in demand. During the last 25 years the meat industry has experienced a gradual but major shift in consumer preference for high fat to leaner and less fat meat and meat products. This preference for leaner meat products has been driven by increased awareness by the public that consumption of excessive dietary fat, particularly saturated fat, may contribute to obesity, cardiovascular diseases, and other fat-associated health problems. As a result of this concern, consumption of red meat products has declined during the last decade but the claimed fat-related health problems by health officials have not improved, which tends to suggest that consumption of red meat products may not be the sole contributor to these health problems. No doubt the meat industry must do everything possible to reduce fat deposition in meat-producing animals if it plans to compete in the market for low-fat products. The meat industry has responded by developing and implementing several strategies to depress fat deposition in meat-producing animals; the most successful strategy to date is the use of large, late-maturing animals for meat production. At desired market weights large-frame, late maturing animals are much leaner compared to small frame, early maturing animals. As it relates to research, animal scientists have investigated the effects of exogenous growth promotants to enhance carcass lean (high protein:fat ratio). The use of steroids such as estrogen, estrogen analogs, and trenbolone acetate [TBA] (approved by the FDA) is effective in ruminants (beef) to improve carcass lean (Hayden et al., 1992). βAA such as clenbuterol (CLEN), ractopamine (RAC), and cimaterol (CIM) have been shown to be effective in increasing lean tissue deposition in non-ruminant animals such as pigs. Furthermore, the antilipogenic properties of exogenous GH have been reported (Harris et al., 1993; Kramer et al., 1993; Liu et al., 1994), although exogenous βAA and GH are less effective in poultry (Johnson, 1989).

β-Adrenergic Agonists and β-Adrenergic Receptors

βAA are structurally similar to the mammalian neurotransmitter epinephrine; they are organic molecules that recognize and bind βAR (see Figure 1 for the chemical structure of commonly used βAA in meat animals). βAR are a member of the seven membrane spanning-receptors; they are glycoprotein receptors that transverse the plasma membrane with a stretch of about 20-25 hydrophobic amino acids with extracellular N-termini involved in ligand binding (Suryanarayana & Kobilka, 1993) and cytosolic C-termini involved in receptor desensitization (Hausdorff et al., 1989) and which interact with G_s of the G-protein (Okamoto et al., 1991). Presently, there are 10 sub-classes of known adrenergic receptors: (a) three distinct Beta-adrenergic receptors (Beta 1, 2, and 3), (b) four Alpha 1-adrenergic receptors (Alpha 1-A, B, C, and D), and (c) three Alpha 2-adrenergic receptors (Alpha 2-A, B, and C), all of which are encoded by different genes.

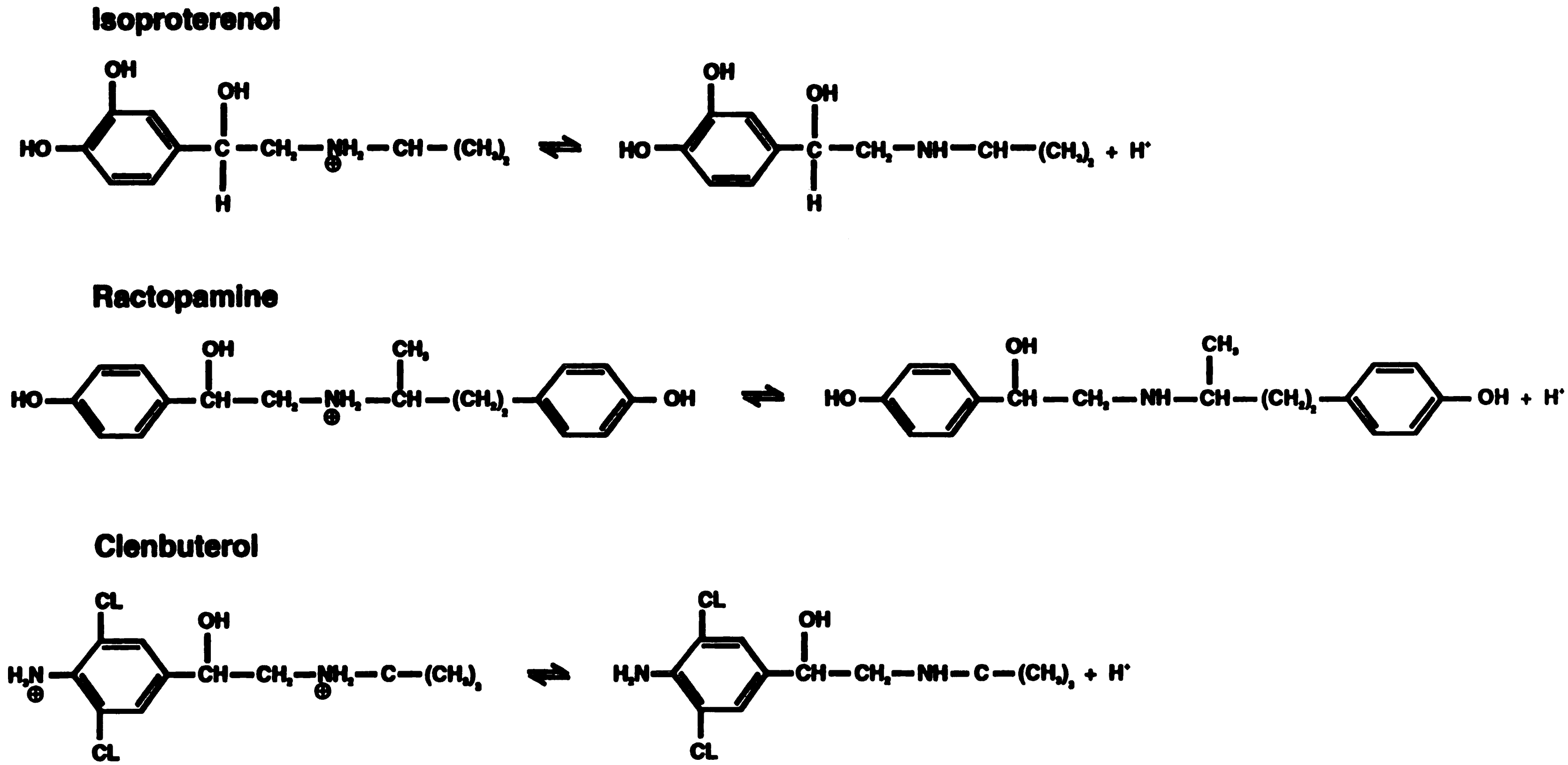
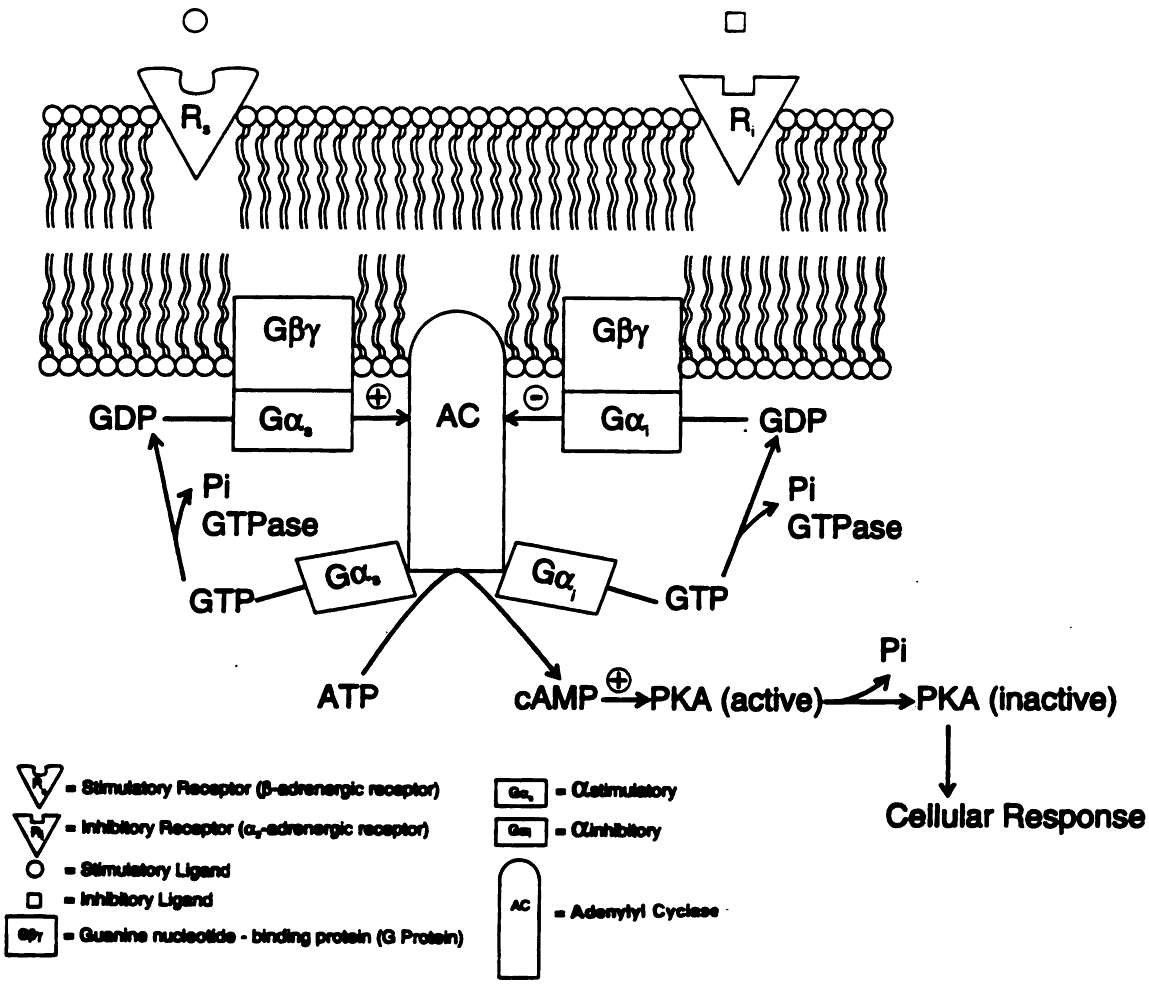


Figure 1. The chemical structure of commonly used βAA in meat animals.

The βAR are coupled to heterotrimeric guanine nucleotide-binding proteins (G-proteins) which are, in turn, coupled to an effector which may be an enzyme (Lefkowitz & Caron, 1988) or ion channel (Reuveny et al., 1994). The G-proteins are composed of three subunits--alpha, beta, and gamma. These three subunits remain associated through the alpha (α) subunit with guanine diphosphate (GDP). This complex interacts with surface receptors (De Lean et al., 1980). The binding of a ligand to the receptor causes the α subunit of the G-protein to exchange guanine diphosphate (GDP) for GTP, resulting in an activated G_α-GTP that subsequently dissociates from the $G_{\alpha\beta\gamma}$-Receptor-Ligand complex to stimulate (reviewed by Lefkowitz & Caron, 1988), or inhibit (Gilman, 1981) adenylyl cyclase. The α subunit may be stimulatory or inhibitory to adenylyl cyclase. The α subunit has GTPase activity that hydrolyzes GTP to GDP+P_i, which means that activation of the G-protein is only transient. Adenylyl cyclase takes ATP as a substrate and generates adenosine 3',5' phosphate (cAMP) as a product (Sutherland & Rall, 1960). Hence the biological consequence of receptor activation is increased intracellular cAMP (see Figure 2 for the mechanism of βAA-induced adenylyl cyclase activation). Cyclic AMP-dependent protein kinase A (PKA) is activated by cAMP association with its regulatory subunits allowing the dissociation of the catalytic subunits of the PKA to phosphorylate its substrates. The substrates for PKA are recognized by a conserved region in the primary sequence (R-R/L-X-S*/T*), where * denotes phosphorylation residue. Phosphorylation catalyzed by PKA elicits a pleiotropic effect in nutrient metabolism and body tissue metabolization in animals. Of course, only agonists of these receptors are capable of producing this βAR-induced cAMP increase.



The $G\alpha_s$-GTP complex stimulates adenylyl cyclase, resulting in an increase in cAMP, while the $G\alpha_i$-GTP complex inhibits adenylyl cyclase, resulting in a decrease in cAMP. Thus, the interplay between the stimulatory and inhibitory complexes modulates the cellular cAMP level.

Figure 2. The mechanism of Beta-Adrenergic Agonist-induced activation of adenylyl cyclase.

The βAR systems have been studied extensively, and thus have become the paradigm for studying signal transduction mechanisms. Since these receptors are the first molecules in the signal transduction pathway, they are subject to several levels of regulation. Some of the regulatory points will be discussed later at the appropriate time in this review of literature. Other signaling pathways involving G-protein are also known. Rhodopsin, for example, is a light-sensitive G-transducin (G_t) coupled receptor that undergoes a conformational change upon activation by light to activate cyclic guanosine monophosphate (cGMP) phosphodiesterase to hydrolyze cGMP (Stryer, 1986). Cyclic GMP phosphodiesterase regulates the opening and closing of the Na^+ Ca^{2+} channels in the plasma membrane of the retinal cells. The muscarinic receptor is also a member of the seven membrane spanning-receptor coupled to a G-protein, G-potassium (G_k), which controls potassium channels (Reuveny et al., 1994). G-olfactory (G_{olf}) is involved with the sense of smell. Of particular interest is Fusin, a novel putative G-protein-coupled receptor with the seven transmembrane segment motif, which is important for HIV-1 infection in CD4-expressing, non-human cells (Feng et al., 1996).

While these receptors are closely related, they are coupled differently. βAR 1, 2, and 3 are coupled to adenylyl cyclase through G-stimulatory (G_s), hence activation of this subclass of receptors by βAA results in elevation of intracellular cAMP levels (Izebvigie & Bergen, 1996a; Izevbigie & Bergen, 1996b; reviewed by Lefkowitz & Caron, 1988). The effects of α 2-receptors are antagonistic to βAR (Coutinho et al., 1993); they are coupled to G-inhibitory (G_i) protein to attenuate intracellular cAMP levels (Gilman, 1987). NAD-dependent ribosylation of the G_s

subunit catalyzed by pertussis toxin produces similar effects by inhibiting the alpha-inhibitory (α_i) subunit of the G-protein. Cholera toxin-catalyzed ADP-ribosylation of the alpha-stimulatory (α_s) inhibits the ATPase activity of the α_s, which sustains α_s activation. Thus both pertussis and cholera toxins lead to elevation of intracellular cAMP (Schramm & Selinger, 1984). Alpha-1 receptors are coupled to phospholipase C (PLC), an enzyme that catalyzes the cleavage of phosphatidylinositol (PI 4,5-bisphosphate) to yield diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) (Kjelsberg et al., 1992). DAG activates phosphokinase C (PKC), an enzyme involved in cell growth and differentiation via the MAP kinase cascade. IP_3 activates the IP_3 receptor, a Ca^{2+} release channel on the endoplasmic reticulum (Furuichi et al., 1989). This activation of the IP_3-gated Ca^{2+} channel leads to increased intracellular Ca^{2+} concentration.

On average, these receptors (βAR) are composed of about 414 amino acids and have a molecular weight ranging from 60,000-80,000 (Benovic et al., 1984; Boege et al., 1988; Lomsney, 1986; Regan et al., 1986). These receptors are pharmacologically distinguishable based on their ligand specificities and affinities, although it is possible, for example, for all sub-types of βAR (βAR 1, 2, and 3) to be recognized by a single ligand. For example, a hydrophilic, non-selective Beta-adrenergic antagonist CGP12177 (Hosada & Duman, 1993) may recognize all three subtypes of βAR (β1-AR, β2-AR, and β3-AR) with different affinities. Differences exist among the βAR. β1-AR has a proline-rich 24-amino acid sequence (PARPPSPSPSPVPAPAPPPGPPRP) present in the third intracellular loop of the receptor, but not present in β2-AR or β3-AR (Green & Liggett, 1994). Proline is known to introduce kinks in proteins, and is thus known as a helix-breaker. Proline is usually found in turns made by polypeptides. The presence of such a proline-rich sequence may confer a rigid conformation in that region of the β1-AR, a region believed to be important for βAR/G_s interaction. Hence β1-AR has a lower efficiency of agonist-stimulated G_s coupling compared to β2-AR (Green & Liggett, 1994). Deletion of the proline-rich region of the β1-AR improved coupling efficiency and ability of the receptor to form a high-affinity ternary complex. Insertion of the proline-rich sequence of β1-AR into β2-AR decreased coupling efficiency of β2-AR (Green & Liggett, 1994). Yet another interesting feature about β1-AR and β2-AR genes is that they are both intronless (Strosberg, 1990), an unusual characteristic of eukaryotic genes. On the other hand, β3-AR is more resistant to desensitization compared to β2-AR because β3-AR has fewer serine/threonine phosphorylation residues available for PKA and beta-adrenergic receptor-associated kinase (βARK) phosphorylation, and hence prolonged stimulation of cAMP production (Liggett et al., 1992) in β3-AR (see Figure 3 for the structure and membrane topology of human β2-AR). This suggests that β3-AR may be important for lipolysis and thermogenesis (Krief et al., 1993); mice with knockout gene (disruption of the β3-AR gene expression) exhibited reduced βAA-stimulated lipolysis (Susulic et al., 1995). Therefore, β3-AR specific agonists may be used as anti-obesity and antidiabetic drugs in humans and anti-obesity drugs in animals (Connacher et al., 1994).

Another important feature about β3-AR is that multiple cAMP response elements (CRE) have been reported (TGACTCCA, TGAGGTCT, and CGAGGTCA located in the 418, 622, and 1125 bases) upstream of the β3-AR coding region. The

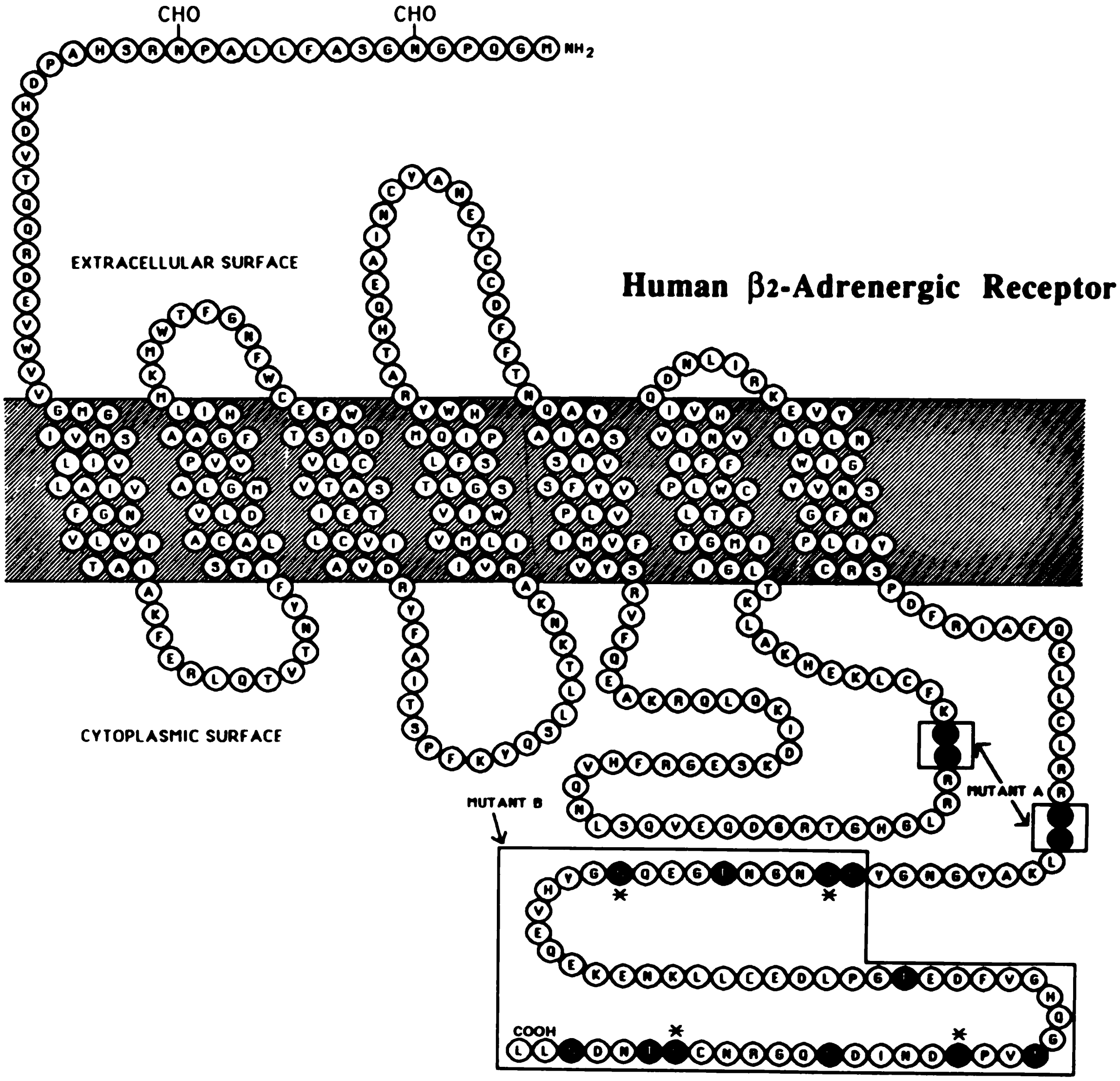


Figure 3. The structure and membrane topology of human β2-AR. (Hausdorff et al., 1989). Reproduced with permission.

biological implication of these CRE is that β3-AR in humans up-regulates its own expression in the presence of intracellular cAMP (Thomas et al., 1992). Also in humans, Collins et al. (1990) reported that CRE enhanced transcription of the human β2-AR. There is evidence of β3-AR in rodent adipose tissue. However, evidence for the presence of β3-AR in adult humans is in dispute (Thomas & Liggett, 1993; Walston et al., 1995; Widen et al., 1995). Some naturally occurring mutations in this receptor have been studied. Trp 64 Arg mutation is associated with obesity and may contribute to the onset of non-insulin-dependent diabetes melitus (NIDDM) (Walston et al., 1995; Widen et al., 1995).

βAR Covalent Modifications

Continuous exposure of βAA to cells containing βAR often results in a rapid desensitization of βAR (Hausdorff et al., 1990, Johnson et al., 1978). Several molecular mechanisms are responsible for the desensitization that is reversible within seconds to minutes (Hausdorff et al., 1990). A rapid receptor desensitization results from two distinct mechanisms of phosphorylation (Hausdorff et al., 1989): PKA, and (βARK). Phosphorylation of the βAR by PKA, βARK, or PKA plus βARK, induces a conformational change that promotes the uncoupling of the βAR-G_s system. PKA-mediated phosphorylation occurs at nanomolar isoproterenol (ISO) while βARK-mediated phosphorylation occurs at micromolar ISO (Hausdorff et al., 1989). βARK-mediated phosphorylation may be of physiological importance in tissues in which βAR are exposed to high concentrations of catecholamines such as neural synapses. PKA-mediated phosphorylation does not require receptor occupancy (Clark et al., 1988). In contrast, βARK-mediated phosphorylation does (Lohse et al., 1989). Historically, receptor desensitization has been described in two distinct ways: heterologous and homologous desensitizations. Homologous desensitization occurs due to loss of receptor responsiveness to a desensitizing agonist, whereas heterologous desensitization is said to occur due to lack of receptor responsiveness to a number of agonists, including the desensitizing agonist. Several lines of evidence have shown the PKA-mediated phosphorylation to be responsible for heterologous desensitization. Liggett et al. (1989) demonstrated that cells expressing the native β2-AR displayed a rapid decline in agonist-induced cAMP accumulation, while the mutant construct lacking the putative PKA phosphorylation site showed prolonged increase of cAMP accumulation (Liggett et al., 1989). Other investigators have also reported that deletion of the PKA putative phosphorylation site in β2-AR inhibited heterologous but not homologous receptor desensitization (Clark et al., 1989). Homologous desensitization is believed to be βARK-mediated based on studies using both wild-type (WT) and Kin^- (S49 lymphoma cells lacking PKA activity). For example, Post et al. (1996) reported similar kinetics of cAMP accumulation and agonist-induced cell surface β2-AR loss in WT and Kin^- S49 lymphoma cells. These data suggest that homologous desensitization may be mediated by a β2-AR specific kinase, possibly βARK, which requires a cytosolic cofactor, Beta-arrestin (β-arrestin), to augment its activity.

βAR Sequestration

Desensitization is believed to precede sequestration (Waldo et al., 1993). Chronic exposure of cells to ligands causes a loss in the ability of the receptor to bind hydrophilic, but not hydrophobic, ligands. This phenomenon is referred to as "sequestration." Evidence suggests that sequestration occurs due to internalization of the surface receptors (Yu et al., 1993). Hydrophobic ligands can translocate the plasma membrane (hydrophobic environment); in contrast, it is energetically unfavorable for hydrophilic ligands to do so. Highly conserved tyrosine residues have been implicated as important for βAR sequestration (Barak et al., 1994). Receptor sequestration or internalization of phosphorylated receptors from cell surface into the cytosol, where phosphatases may be located, promotes dephosphorylation of the phosphorylated receptors so that they may be recycled (Zastrow & Kobilka, 1992). Blocking sequestration by pretreating cells with pharmacological agents such as concanavalin A or phenylarsene oxide had no apparent effect on rapid desensitization (Waldo et al., 1983). This suggests that βAR desensitization does precede sequestration of receptors.

βAR Isomerization

βAR may assume active and inactive conformations. The binding of agonists may promote the formation of active conformations capable of interacting favorably with G_s of the G-protein (Kjelsberg et al., 1992), whereas the binding of antagonists may promote formation of a partially active or inactive conformation that is incapable of interacting with G_s, hence producing no biological consequences. βAA interact with βAR to form βAA-βAR complexes whose stability depends on the affinities of the agonists for the receptors. Tight binding, referred to as low K_D or K_i, is usually indicative of a stable binding, but not of the ability to induce a biological response (Coutinho et al., 1990; Liu & Mills, 1989). A wild type β2-AR recognizes and binds propranolol and dihydroalprenolol hydrochloride (DHA) as agonists. The amino acid ASN312 is important for ligand binding by participating in hydrogen bond formation with the phenoxy oxygen provided by propranolol and DHA. Substitution of ASN312 with amino acids that cannot form hydrogen bonds (alanine and phenylalanine) prevented binding of the compounds (Suryanarayana & Kobilka, 1993).

Furthermore, substitution of ASN312 with glutamine and threonine enabled other compounds to act as agonists (Suryanarayana & Kobilka, 1993). Some naturally-occurring polymorphisms have been reported to have occurred in the region of the receptor crucial for the formation of stable agonist-receptor-G_α complexes; serine 164 of the human β2-AR in the fourth transmembrane domain, purported to be the ligand binding pocket, interacts with the β-carbon of adrenergic ligands. Mutations at serine 164 to Ile decreased binding affinity (1450$\pm$79 versus 368$\pm$39 nM), adenylyl cyclase stimulation, and βAR sequestration (Green et al., 1993). Ligands lacking the hydroxyl groups on their β-carbon were not affected by the serine 164 to Ile polymorphism (Green et al., 1993). Agonist-receptor complexes may interact with specific G-proteins (Jones & Read, 1987). Studies with rat olfactory neuroepithelium indicated the existence of multiple forms of $G_{\alpha s}$ (Jones & Read, 1987). The biological implication of multiple $G_{\alpha s}$ species is that there may be no additive response when two agonists coupled to the same G-protein are used simultaneously. For example, in the hepatocytes derived from partially heptectomized male rats, epinephrine and glycogen were found to be coupled to the same $G_{\alpha s}$ system (Yagami, 1995). However, the potency of activation for each agonist may be different. Furthermore, when multiple agonists are used concurrently, one agonist may affect the affinity of the other (Yagami, 1995). These data might help to explain, at least in part, why Liu and Mills (1989) observed a concentration-dependent inhibition of epinephrine-stimulated lipolysis by RAC or CLEN in pig adipocytes. On this basis, some investigators suggest that in vivo RAC and CLEN may block the lipolytic responsiveness of the βAR-adenylyl cyclase systems to catecholamines. The preponderance of evidence from in vivo (Bergen et al., 1989; Grant et al., 1993; Helferich et al., 1990) and in vitro studies (Anderson et al., 1990) showed that βAA increased lean tissue deposition while decreasing adipose tissue deposition (Barak et al., 1992). However, the magnitude of responses vary between laboratories; the reasons for some of the variations may be due to tissue-specific responses to agonists (Spurlock et al., 1994) species-specific responses to agonists (Mersmann, 1984), and pharmacodynamics of the agonists. Therefore, data obtained using a particular species and agonist must not be extrapolated across species. An intriguing question would be what properties make a given drug an agonist or antagonist. The answer is unclear, but it is known that the ability of some agonists to bind tightly to their receptors is unrelated to their ability to stimulate lipolysis (Liu & Mills, 1989). Rather, the primary distinguishing property between beta-agonist and antagonist resides in their ability to activate adenylyl cyclase to produce cAMP (Jasper et al., 1988).

β-Adrenergic Agonists: Effect on Lean Tissue Deposition

Skeletal muscle is a major target for βAA actions. βAA-fed animals deposited more lean tissue compared to the control (Bergen et al., 1989; Grant et al., 1993) although the magnitude of the βAA-induced hypertrophy depends on dietary protein intake (Adeola et al., 1990; Adeola et al., 1992; Bergen et al., 1989). The lean tissue-stimulatory effect of βAA was also demonstrated in vitro; this effect was reversed in the presence of propranolol, a beta blocker or antagonist (Anderson et al., 1990). This tends to suggest that the effects of βAA on skeletal muscle growth may be βAR-mediated (Anderson et al., 1990; Choo et al., 1992); however non-βAR-mediated βAA actions have been proposed (Bergen et al., 1989; Smith, 1989). The mechanism whereby βAA promote lean tissue deposition is a matter of controversy. The mechanism may be via increased protein synthesis (Bergen et al., 1989; Grant et al., 1993; Helferich et al., 1990) or increased expression of certain protease inhibitors to decrease protein degradation (Pringle et al., 1993).

When protein synthesis rate was measured in vivo by continuous infusion with [^{14}C] lysine, Helferich et al. (1990) reported that the fractional synthesis rate (FSR) of skeletal muscle α-actin was enhanced by 55% in RAC-treated pigs compared to the control. Bergen et al. (1989) observed an increase in fractional accretion rate (FAR) due to increased FSR in the semitendinosus muscle of barrows. These data generated from continuous infusion of radioactive amino acids further underscores the lean tissue-enhancement properties of βAA. Liu et al. (1994) reported that RAC increased nitrogen retention in longissimus muscle area, and increased α-actin gene expression.

β-Adrenergic Agonists: Effects on Adipose Tissue Deposition

Depressed body fat deposition is one of the metabolic consequences of feeding βAA to animals. βAA depress fat deposition by decreasing lipogenesis through the inhibition of lipogenic enzymes (Dickerson, 1990). Furthermore, βAA induced cAMP release which activate cAMP-dependent protein kinase A (PKA) which, in turn, activate or deactivate a series of intracellular enzymes by phosphorylation including hormone-sensitive lipase. Increased lipolysis is due to increased in triacylglycerol lipase activity (Yang & McElligott, 1989). Studies with pigs showed that RAC enhanced lipolysis, while adipose tissue malic enzyme fatty acid synthase activities were decreased (Merkel et al., 1987) (see Figure 4 for the schematic diagram of βAA action on various tissues). Evidence from in vitro studies indicates that βAA stimulated glycerol release and inhibited fatty acid synthase (FAS) activity in a dose-dependent manner in TA1 cells (Dickerson-Weber et al., 1992). In pig adipose tissue explants, Peterla and Scanes (1990) reported that ISO, CIM, and RAC

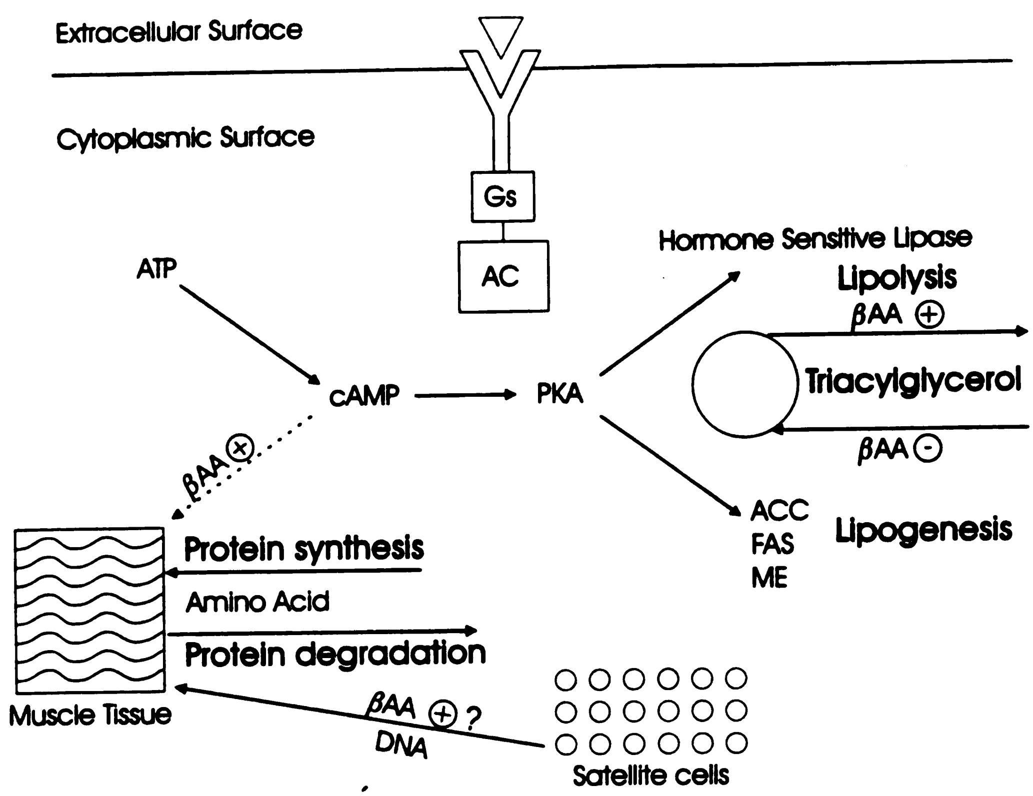


Figure 4. Schematic diagram of βAA action on various tissues.

exhibited lipolytic and antilipogenic effects. Pigs fed diets supplemented with 20 ppm RAC showed higher blood non-esterified fatty acids (NEFA) compared to the control (Adeola et al., 1992). These results underscore the contention that βAA depress fat deposition in animals by increasing the lipolytic activity (Adeola et al., 1992; Merkel et al., 1987; Peterla & Scanes), while decreasing lipogenesis (Dickerson, 1990; Merkel et al., 1987; Peterla & Scanes, 1990).

Exogenous Growth Hormone (GH): Effect on Lean Tissue Deposition

Administration of exogenous GH to animals has resulted in improved protein:fat ratio and feed efficiency (Caperna et al., 1995; Grant et al., 1991; Solomon et al., 1988; Verstegen et al., 1990). The effective dose range of GH is 50-100 μg/kg BW (Thiel et al., 1993). Although animals must be fed not only adequate dietary crude protein to show growth hormone-stimulated muscle hypertrophy (Seve et al., 1993; Solomon et al., 1988), but lysine, a first limiting amino acid in pigs' diet, must be no less than 1.2% (Hansen et al., 1994). Pigs injected with porcine somatotropin (pST) had improved feed efficiency up to 1.2% lysine (Hansen et al., 1994).

Growth hormone-treated pigs tend to have heavier livers, kidneys, and hearts (Fabry et al., 1991). It is not clear whether GH stimulates the growth of these organs directly or indirectly as a result of improved growth rate. In pigs, GH increases protein synthesis by enhancing the rate of amino acid incorporation into proteins (Caperna et al., 1995) and fractional synthesis rate (FSR) as measured by flooding dose of L-[1-13C] valine (Seve et al.,1993). The mechanism of GH-mediated muscle hypertrophy is not clearly understood. However, there is a positive correlation between GH administration and liver and adipose tissue IGF-1 mRNA (Coleman et al., 1994; Grant et al., 1991). Hence the GH effects on these organs and tissues may be IGF-1-mediated. However, in skeletal muscle, GH and IGF-1 mRNA are not correlated, thus suggesting that the GH effects on IGF-1 gene expression may be tissue specific (Coleman et al., 1994).

Exogenous Growth Hormone (GH): Effect on Fat Deposition

The effects of GH oppose those of insulin on lipid and carbohydrate metabolism and may be called counter-regulatory hormone (Davidson, 1989). In barrows, administration of exogenous GH resulted in reduced acetyl CoA carboxylase (ACC) enzyme activity, and protein content and mRNA abundance for ACC in adipose tissue by 40 to 50% (Liu et al., 1994). Grant et al. (1991) and Coleman et al. (1994) reported an elevated IGF-1 mRNA level in the liver, and an increased serum IGF-1 concentration following GH treatment. Subcutaneous adipose tissue IGF-1 mRNA level was increased in GH-treated pigs (Coleman et al., 1994). Based on these findings, Coleman et al. (1994) concluded that GH actions on adipose tissue may be IGF-1 mediated. GH-treated barrows showed a 79% decrease in total activated ACC and a 67% decrease in fatty acid synthase (FAS) activity (Harris et al., 1993). Furthermore, northern blot analysis indicated a 90% decrease in the FAS mRNA (Harris et al., 1993). Mildner and Clark (1991) used a 1.5-Kb cDNA probe, representing the thioesterase domain of the multi-functional porcine FAS to examine the tissue distribution of FAS mRNA within the pig and concluded that porcine GH significantly depressed FAS mRNA in both adipose tissue and liver. It has been suggested that porcine GH may also reduce fat deposition in growing pigs up to 70% by inhibiting lipogenesis and the expression of insulin-responsive gene such as glucose transporter protein 4 (GLUT 4) (Etherton et al., 1993). Taken together, GH depresses fat deposition by decreasing lipogenesis through pretranslational regulation of lipogenic enzymes: ACC (Harris et al., 1993; Liu et al., 1994), FAS (Harris et al., 1993; Mildner & Clark, 1991), and GLUT 4 (Etherton et al., 1993).

Additive Effects of βAA and GH

Research data suggest that βAA are effective in promoting lean tissue deposition (Bergen et al., 1989; Grant et al., 1993; Helferich et al., 1990). The FSR of skeletal muscle α-actin was enhanced by 55% in RAC-treated pigs (Helferich et al., 1990) and GH more effective in reducing fat deposition. In growing pigs, fat deposition may be decreased up to 70% by GH (Etherton et al., 1993). In 1991, a GH-adipose tissue membrane G_i protein complex was reported (Roupas et al., 1991). $G\alpha_i$ interacts negatively with adenylyl cyclase to attenuate the level of cellular cAMP. Whereas βAA-induced $G\alpha_s$ dissociation from the heterotetrameric G protein stimulates adenylyl cyclase. Thus the interplay between the $G\alpha_s$ and $G\alpha_i$ modulates the level of cellular cAMP. GH hormone may nullify the attenuation function of the G protein to augment cAMP production (Roupas et al., 1991). Therefore further stimulation of the G protein by βAA may further increase cellular cAMP level. Knowing this, one may envisage that concomitant administration of βAA and GH may result in additive effects. Indeed it does (Hansen et al., 1994).

Of interest is the biochemical evidence that muscle and adipose tissues possess βAR which may be activated by βAA. Spurlock et al. (1994) evaluated the effect of feeding RAC, a phenethanolamine, on beta-adrenergic receptor densities and affinities in both adipose and muscle tissues. The investigators reported that RAC did not affect the maximum binding (B_{max}) of [^{3}H]dihydroalprenolol ([^{3}H]DHA) to longissimus muscle membrane preparations. However, in the adipose membrane preparations, RAC reduced β-adrenoceptor density by approximately 50%. Furthermore, RAC feeding did not affect [^{3}H]DHA affinity for βAR in muscle or adipose tissue. In another experiment Spurlock et al. (1993) studied the affinities (K_i) with which CLEN, RAC, and L-644,969 bind βAR population in porcine adipose and muscle membranes in the presence of a competitor [^{3}H]DHA. CLEN had the highest affinity, 125 nM (the lowest K_i), followed by L-644,969 (350nM), and RAC (856 nM). K_i values were similar within a given agonist regardless of tissue type, except that RAC had a higher affinity, 856 nM, for the middle subcutaneous (SQ) adipose tissue. Coutinho et al. (1992) reported the presence of two subtype βAR (β_1-AR and β_2-AR) in porcine adipocyte crude membrane preparations of which 45% of the receptors had a high affinity for ICI89,406, a β_1-AR antagonist, $K_i = 2.27 \pm 0.68$ nM.

Muscle Biology

Skeletal muscle growth is of great interest to animal agriculture because muscle represents the most economically important tissue in the animal's body. In the human diet, muscle serves as a source of minerals, vitamins, and high quality protein. For these reasons, animal scientists continue to investigate factors that regulate prenatal and postnatal skeletal muscle growth in their quest to maximize lean tissue deposition while minimizing fat deposition.

Embryonic myoblasts arise from mesodermal cells. These stem cells are capable of giving rise to either adipoblasts, chondroblasts, myoblasts or osteoblasts. Various growth factors take part in the determination of the fate of stem cells. The process by which myoblasts arise from mesoderm cells is termed "determination," which results in a population of proliferative cells and their descendants committed to the myogenic lineages (reviewed by Stockdale, 1992). During embryonic development of muscle tissue, two morphologically distinct myotubes, primary and secondary, form. Both contain mononucleated cells enclosed by a common lamina. The secondary myotube forms on the surface of the primary myotube and later become free of the primary myotube. Consequently, non-fused mononucleated cells are trapped between the basement membrane and sarcolemma. Myogenic cells proliferate, differentiate, and subsequently fuse with each other or myotubes to become a multi-nucleated myotube. Since fused nuclei in multinucleated myotubes are mitotic-incompetent, how then is the increase in DNA and protein concentrations observed in hypertrophied skeletal muscle accounted for?

Satellite Cell Effects on Skeletal Muscle Growth

In most species, including meat-producing animals, myotube formation is essentially complete at birth or shortly after. Therefore, beyond birth, muscle fiber number remains virtually constant. During skeletal muscle growth, myofibers increase in size due to either hyperplasia (increase in cell number) or hypertrophy (increase in cell size). The latter is mostly responsible for postnatal muscle growth indicated by increased protein:DNA and protein:RNA ratios (Skjaerlund et al., 1994). As a common feature among cells, genes need to be transcribed to yield total RNA, and mRNA must be translated to yield proteins required for structural or enzymatic function in the cell. Protein accretion is a dynamic process that is dependent on protein synthesis and degradation. Protein accretion occurs when synthesis is greater than the rate of degradation, as is typical in growing animals, but when protein degradation is greater than synthesis, muscle mass degeneration occurs. Comparing the longissimus dorsi (LD), semitendinosus (ST), and brachialis (BR) muscle fractional synthesis rate (FSR) of 45 kg (older) versus 22 kg (younger) pigs, Mulvaney et al., (1985) reported a 20% lower fractional synthesis in the 45 kg pigs compared to the 22 kg pigs. However, the fractional breakdown rate (FBR) derived by difference (FBR = FSR - FAR [fractional accretion rate]) was lower in the older pigs, suggesting muscle growth rate may be modulated by alterations in FBR (Mulvaney et al., 1985). Since myofibers are mitotic-incompetent, this means the number of nuclei present within each myofiber remains constant. An intriguing question then arises: How then are the nuclei, whose numbers remain constant, able to meet the protein synthesis needs of the increasing cytoplasmic volume? CNR, as expressed by Landing et al. (1974), is the cytoplasmic volume to nucleus ratio. Postnatal muscle growth is largely due to increasing CNR (Mozdziak et al., 1994), thus suggesting little or no change in the number of nuclei present in the myofiber. In contrast, Moss (1968) reported that the DNA unit size (which is the same as CNR) remains constant, indicating that, as fibers undergo hypertrophy, they accumulate more proteins, and DNA from outside source must be added to myofiber in order to maintain a constant CNR. Seven years earlier Mauro (1961) had described satellite cells as mononucleated, mitotic-competent cells trapped between the sarcolemma and basement lamina of the muscle fiber, indicating that satellite cells are the source of nuclei or DNA addition to myofibers during postnatal muscle growth (Allen et al., 1979) or regeneration (Mauro, 1961). Satellite cells are myogenic cell precursors; they proliferate, differentiate to become myogenic cells that subsequently fuse either with each other to form new myofibers, or fuse with existing myofibers. It is now fairly well-established that satellite cells contribute nuclei, and consequently more DNA and protein, to myofibers (Appell et al., 1988; Kennedy et al., 1988). Mozdziak et al. (1994) observed an age-related decrease in satellite mitotic activity, and also an age-related increase in CNR in turkey satellite cells. These data suggest that satellite proliferation, differentiation and subsequent fusion are more important for growing animals, while late-phase postnatal growth is largely due to hypertrophy (Mozdziak et al., 1994). Since satellite cells play a key role in postnatal muscle growth and development, it becomes imperative to understand the factors involved in the activation of satellite cells from the quiescence stage to proliferation, differentiation, and fusion, especially meat animal-derived satellite cells. Optimum conditions for isolation, proliferation, and differentiation for porcine primary satellite cells have been described (Doumit & Merkel, 1992).

In cell culture studies, Doumit et al. (1993) examined the mitogenic properties of basic fibroblast growth factor (bFGF), insulin-like growth factors (IGF-I and II), platelet-derived growth factors (PDGF-AA and BB), and epidermal growth factor (EGF)--individually and combined--in basal serum-free medium or minimum essential medium containing 2% fetal bovine serum (MEM-2% FBS). Individually, bFGF, IGFs, and PDGF-BB stimulated the proliferative activity of porcine satellite cells propagated in basal serum free medium or MEM-2% FBS. EGF promoted the proliferative activity of porcine satellite only in MEM-2% FBS (Doumit et al., 1993). Any combination of bFGF, IGF-I, EGF, and PDGF-BB, except EGF and bFGF, produced a synergistic response (Doumit et al., 1993). Transforming growth factor-beta (TGF-β) may promote or inhibit the proliferative activity of porcine satellite cells depending on the presence or absence of other growth factors (Cook et al., 1993). TGF-β inhibited PDGF-BB-stimulated proliferation, enhanced bFGF-stimulated proliferation, but had no effect on IGF-stimulated proliferation of porcine satellite cells grown in serum-free medium (Cook et al., 1993). When two growth factors were used concomitantly, TGF-β depressed PDGF-BB and IGF-I, PDGF-BB and EGF, PDGF-BB and bFGF, and IGF-I and EGF-proliferative activities of porcine satellite cells (Cook et al., 1993), but TGF-β had no effect on the IGF-I and EGF-stimulated proliferation of porcine satellite cells (Cook et al., 1993). These investigators concluded that bFGF and TGF-β interact favorably to increase the bFGF-stimulated proliferative activity while TGF-β interacts unfavorably with PDGF-BB to depress the mitogenicity of PDGF-BB. Taken together, bFGF, IGF_{s}, TGF-β, and PDGF-BB are potent regulators of porcine satellite cells. Therefore, alteration in the levels of these growth factors remains a possible mechanism to regulate porcine satellite cell proliferative activities. Similar results have been reported in other species such as chicken and rats.

In cultured chicken breast satellite cells, RAC or ISO doubled myotube nuclei number compared to untreated cells; however, the βAA-induced increase in myotube nuclei number was decreased by about 25% in the presence of 10^{-5} M propranolol, a beta-adrenergic antagonist (Grant et al., 1990). FGF-stimulated the proliferative activity of chicken satellite cells (Grant et al., 1990).

In rat studies, Bischoff (1990) reported that mitogens released from injured muscle produced a long-lasting effect that committed dormant satellite cells to proliferate, while serum growth factors were needed to maintain progression through the cell cycle (Bischoff, 1990).

Four myogenic basic helix-loop-helix proteins--myogenin, MyoD, Myf-5, and MRF4--are capable individually to stimulate cell differentiation when introduced to non-myogenic cells (reviewed by Rawls et al., 1995). Gene knockout experiments showed that no more than two--MyoD and myogenin, or Myf-5 and Myogenin--are required for muscle differentiation, at least in rat studies (Rawls et al., 1995). Because in normal, uninjured adult muscle, satellite cells are mitotically quiescent, in the recent years researchers have sought to examine the physiological cues that activate satellite cells from dormant stage to proliferation, differentiation, and subsequent fusion with other satellite cells or myofibers. They have also examined the role of each myogenic factor (Myogenin, MyoD, Myf-5, and MRF4) by monitoring the time of appearance of each myogenic factor during cell culture. In rat satellite cells derived from injured tissues, the first indication of myogenic cell differentiation, an increase in myogenin mRNA expression, occurred within 4-8 h after injury (Rantanen et al., 1995). Furthermore, the first desmin-, $MyoD_1$-, and myogenin-positive myoblasts were observed after 12 h, but satellite cell proliferation, as measured by bromodeoxyuridine incorporation, was not seen until 24 h (Rantanen et al., 1995). This schedule of events (differentiation preceding proliferation) which contradicts the general concept that proliferation precedes differentiation, led these investigators to propose that there may be two populations of satellite cells: (a) One population differentiates immediately following injury, and (b) the other population proliferates (Rantanen et al., 1995). These findings may have been corroborated by Yablonka-Reuveni and Rivera (1994). Using immunohistochemical techniques, Yablonka-Reuveni and Rivera (1994) reported that only half of myogenin or alpha-smooth muscle actin (alpha SM actin) positive adult rat satellite cells were positive for developmental sarcomeric myosin, a differentiation index. These results suggest that only a fraction (about 50%) of the satellite cell descendants entered the phase of terminal differentiation (Rantanen et al., 1995; Yablonka-Reuveni & Rivera, 1994).

Taken together, this literature review suggests that the use of exogenous growth promotants such as βAA and GH (pending FDA approval) in combination with current industrial practices, may result in the production meat products in which fat supplies less than 30% of their total calories. However, consumer acceptance of the use of endogenous agents leads to the following issues: (a) safety, and (b) quality and palatability of meat derived from βAA-treated animals.

In terms of safety, because of the low concentration of βAA required to manipulate carcass composition coupled with an adequate pre-slaughter withdrawal period, it is prudent to speculate that there is little or no risk associated with consuming meat or meat products from βAA-treated animals. Indeed, studies with turkeys indicated that RAC was rapidly eliminated after oral dosing (Smith et al., 1993).

CHAPTER III: CHARACTERIZATION OF CLONALLY-DERIVED M2 PORCINE SATELLITE CELLS--PROLIFERATION AND DIFFERENTIATION STUDIES

Abstract

Data generated from both growth studies quantifying DNA amount or cell number produced similar results. Insulin- or insulin and ara-c-stimulated differentiation, as measured by creatine kinase, were numerically similar and higher than the lower creatine kinase activity observed in ara-c-stimulated differentiation. Ara-c inhibits myoblast DNA synthesis (Turo & Florini, 1982). Insulin addition to the cultures may have countered these effects. DNA content per M2 satellite cell was estimated to be 8.9 pg using procedures described by West et al., (1985).

Introduction

Optimum conditions for the propagation of clonally-derived (M1) porcine satellite cells have been described (Doumit & Merkel, 1992; Merkel et al., 1993). When M1 porcine satellite cells were cultured either in chemically-defined or serum-containing media, they proliferated, differentiated and subsequently fused with each other to form multi-nucleated myotubes (Doumit, 1994; Doumit & Merkel, 1992) that expressed some muscle-specific proteins including myosin and creatine kinase protein (Doumit, 1994). The induction of creatine kinase activity may be used as a marker for myogenic cell differentiation (Turo & Florini, 1982). M2 porcine satellite cells used in these studies, not previously characterized, were generously provided by Dr. Matthew Doumit, USMARC, NE. Both differentiated and non-differentiated M2 porcine satellite cells were utilized in the present studies. The objectives of these studies were: (a) to characterize the proliferation rate of these cells by using a hemocytometer, and DNA quantitation as described by West et al. (1985); and (b) to conduct differentiation studies--creatine kinase activity--units/mg DNA.

Experimental Procedures

Materials and Methods

Antibiotic-Antimycotic (ABAM), gentamicin, Minimum Essential Medium (MEM), and Fetal Bovine Serum (FBS) were purchased from GIBCO BRL (Grand Island, NY). Tissue culture (35 mm diameter, 6-well) plates were purchased from Corning Glass Works (Corning, NY). Bovine pancreas insulin (I-1882), bovine transferrin (T-8027), MCDB-110 medium (M-6520), dexamethasone (D-8893), bovine serum albumin (BSA-RIA grade A-7888), water-soluble linoleic acid (L-5900), porcine skin gelatin (G-1890), bisbenzamide [Hoescht 33258 (B1155)], calf thymus DNA (D-8661), UV-47 kit, cytosine β-D-Arabinofuranoside (C-1768), trypsin-EDTA, and Giemsa stain (G-4507), were obtained from Sigma Chemical Company, St. Louis, MO.

Clonally-derived M2 porcine satellite cells previously isolated by Doumit and Merkel (1992) from the semimembranosus muscle of 6- to 8-wk-old pigs were extended to the fifth passage and used in these studies. Cells were suspended in MEM containing 10% FBS, 0.5% ABAM, and 0.1% gentamicin, seeded at a density of 10,000 cells per 35 mm diameter well previously coated with 0.1% gelatin as described by Richler and Yaffe (1970), and propagated in a humidified CO_2 incubator containing 95% air and 5% CO_2 at 37°.

Cell Number Determination

Cells were grown as described in the Materials and Methods section for 8 d. At 24-h intervals six wells were randomly selected for cell number determination. Medium was aspirated from monolayers, and cell monolayers were then washed with PBS (pH 7.4) and trypsinized. Cells were recovered by centrifugation (1400 x g) for 3 min. Pellets were resuspended to 400,000 cells/mL before counting using a hemocytometer.

DNA Determination

For DNA quantitation, cells were propagated as described in the Materials and Methods section. At 24-h intervals, six wells were randomly selected and assayed for DNA content as described by West et al. (1985) based on the measurement of the relative fluorescence of the DNA-bisbenzamide complex. The amount of DNA per cell was estimated by measuring the relative fluorencence of known cell quantities using calf thymus DNA as a standard.

Satellite Cell Differentiation

Upon confluence at approximately day 5, cells were switched to a serum-free medium as described by Merkel et al. (1993) except bFGF and PDGF-BB were not added, and 10^{-10} M dexamethasone was used, to induce differentiation. This medium was originally formulated to promote growth, but was later modified to promote differentiation (upon verbal discussion with Dr. Doumit). The components of the differentiation-promoting medium are listed in Table 1.

Table 1. Porcine satellite cell serum-free medium.

Component	Final Concentration
MEM:MCDB-110 medium	4:1
Dexamethasone	10^{-10} M
Bovine Serum Albumin	0.5 mg/mL
Insulin	10^{-6} M
Transferrin	100 μg/mL
Water-soluble linoleic Acid	0.5μg/mL

Cells were fed serum-free medium, supplemented with or without 10^{-5} M ara-c for 72 h, and then switched to MEM + 10% FBS (growth medium) for 24 h to support the completion of the myotube formation process. At 24-h intervals, six wells were randomly selected and medium aspirated from monolayers. Cells were washed three times with cold PBS (pH 7.4) and overlaid with 0.5 mL of 0.05 M glycylglycine buffer (pH 6.75). Cells were kept at -20° C for creatine kinase activity analysis (within 5 d) using 47-UV kit (Sigma) based on the procedure described by Szasz et al. (1976) except a 40 μL sample was used for each assay. Duplicate samples of 100μl were taken from each well for DNA content determination using calf thymus DNA as a standard. DNA content per well was determined as described by West et al. (1985) except a 100 μL sample was used for each assay.

Results

Cell Number Determination

Results of the cell quantitations studies are presented in Figure 5. Each data point represents the mean of six incubations.

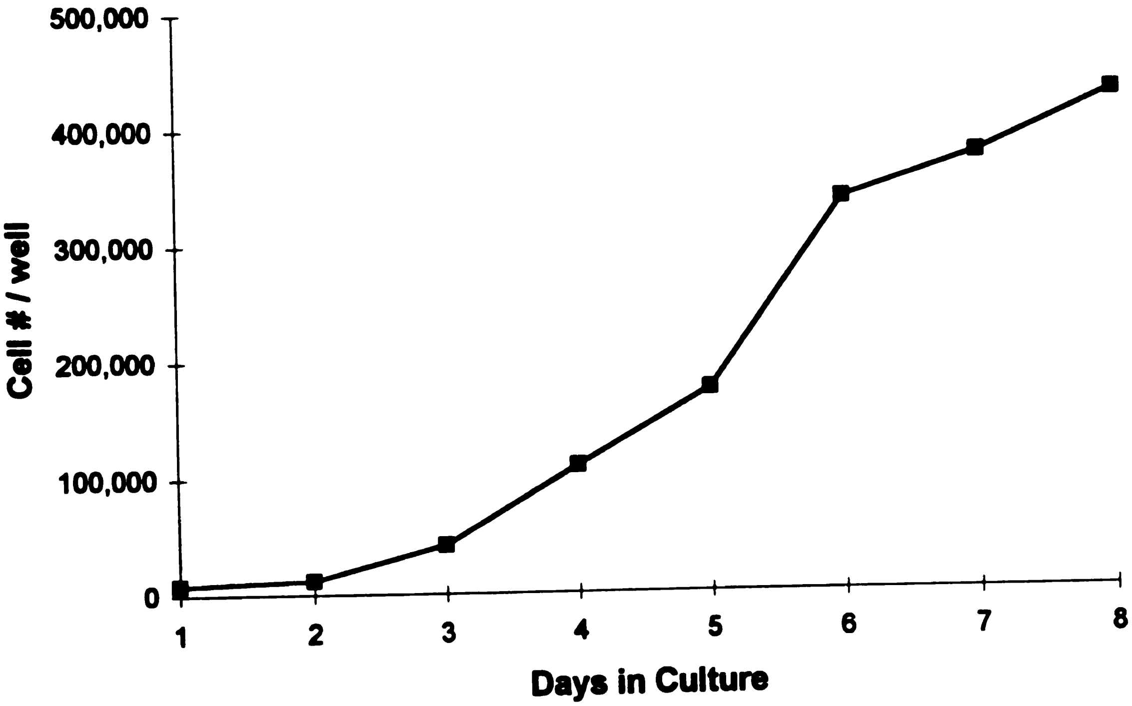


Figure 5. Cell quantitation studies.

DNA Determination

Results of DNA quantitation are shown in Figure 6. Each data point represents the mean ± SD of six incubations.

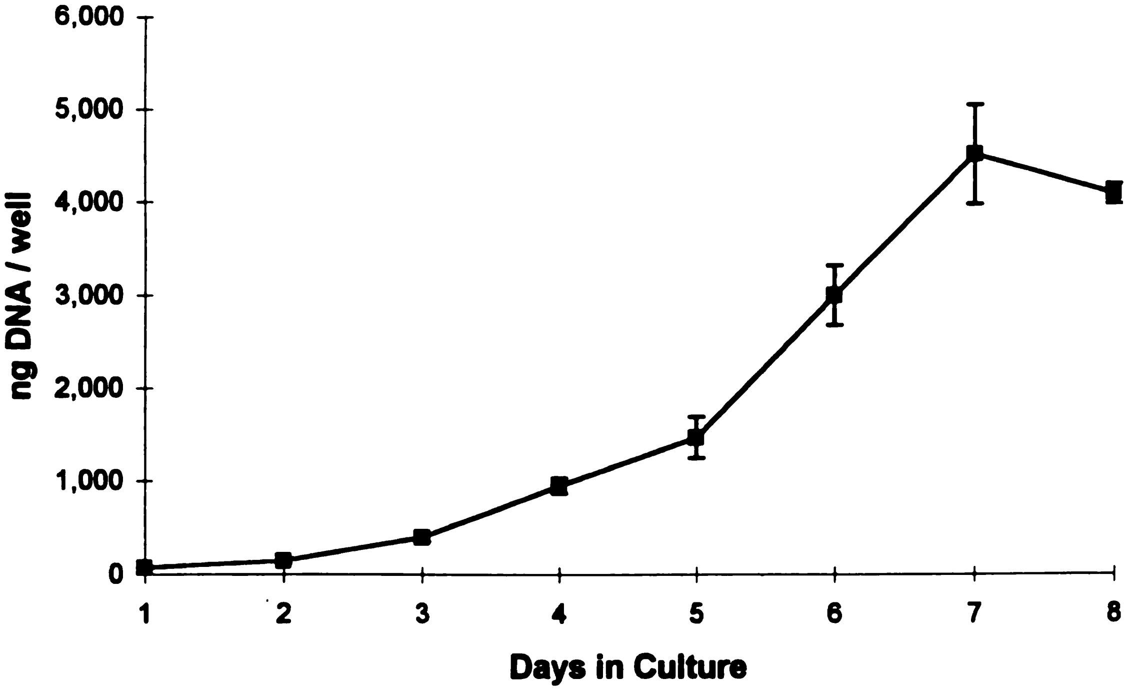


Figure 6. DNA quantitation studies.

Satellite Cell Differentiation

Results of the differentiation studies are shown in Table 2. Data are means ± SD of six incubations. Porcine satellite cells were grown as described in the Materials and Methods section. Cells were differentiated in serum-free medium supplemented with or without 10^{-5} cytosine β-D-arabinofuranoside (Ara-c). For the Ara-c-only treatment, serum-free medium was not supplemented with insulin.

Table 2. Effects of cytosine β-D-Arabinofuranoside on insulin-stimulated M2 porcine satellite cell differentiation.

		Creatine kinase activity (unit/mg DNA)			
Hours since differentiation induction	Day in culture	Prediffer-entiation	Insulin	Insulin + Ara-c	Ara-c
0	5	3.94 ± 0.77	–	–	–
24	6		6.14 ± 1.5	6.94 ± 1.21	4.29 ± 1.18
48	7		6.02 ± 0.9	6.24 ± 2.67	4.68 ± 1.07
72	8		5.15 ± 2.16	4.54 ± 1.24	1.85 ± 0.83
96	9		14.0 ± 7.39	12.55 ± 3.12	11.51 ± 2.35

Figure 7 illustrates the morphology of differentiating satellite cells (early stage) in serum-free medium. Cells were grown in MEM + 10% FBS to confluence at 5 d and cells were placed in serum-free medium (differentiation medium) for 24 h.

Figure 8 illustrates the morphological appearance of differentiating satellite cells (mid-stage) in serum-free medium. Cells were grown in MEM + 10% FBS to confluence at 5 d and cells were placed in serum-free medium (differentiation medium) for 48 h.

Figures 9 and 10 illustrate insulin-stimulated porcine satellite cell differentiation. Figure 9 shows multinucleated myofibers grown in an insulin-only medium. Cells were grown as described in the Materials and Methods section. Upon confluence, cell differentiation was induced by switching cells to a serum-free medium containing 10^{-6} M insulin. Cells were incubated in serum-free medium for 72 h. They were then fixed in absolute methanol and stained with 0.03% Giemsa to visualize the nuclei.

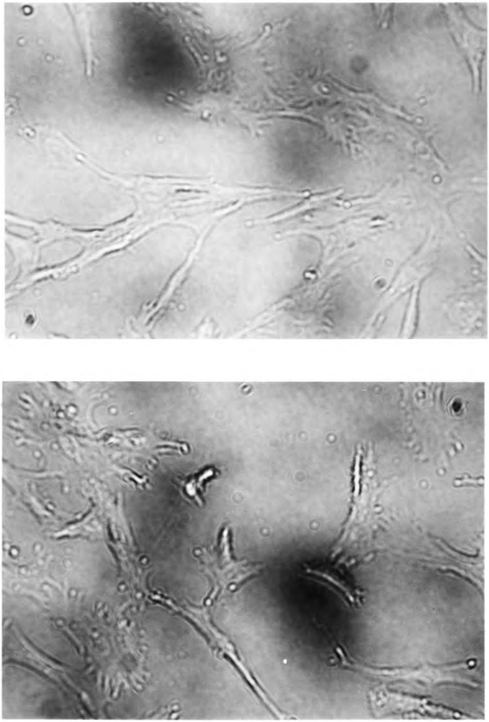


Figure 7. Early-stage differentiation of M2 porcine satellite cells.

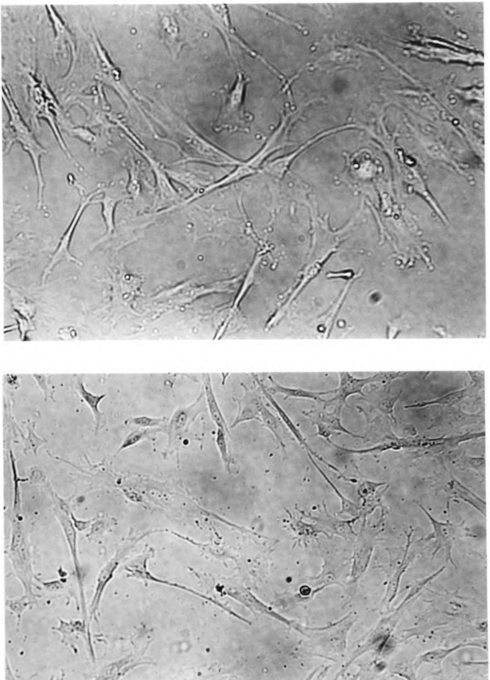


Figure 8. Mid-stage differentiation of M2 porcine satellite cells.

Figure 9. Cells grown in a 10^{-6} M insulin-only serum-free medium, after 72 h of incubation.

Figure 10 illustrates multinucleated myofibers grown in an insulin and ara-c medium. Cells were propagated as described in the Materials and Methods section Upon confluence, cell differentiation was induced by switching cells to a serum-free medium containing 10^{-6} M insulin and supplemented with 10^{-5} M ara-c. Cells were incubated in serum-free medium for 72 h. Cells were fixed in absolute methanol and stained with 0.03% Giemsa to visualize the nuclei.

Figure 11 illustrates the effect of cytosine-β-D-arabinofuranoside on porcine satellite cell differentiation in serum-free medium. Cells were grown as described in the Materials and Methods section. Upon confluence, cell differentiation was induced by switching cells to a serum-free medium with 10^{-5} M ara-c. Cells were differentiated in serum-free medium without 10^{-6} M insulin for 72 h, then fixed in absolute methanol and stained with 0.03% Giemsa for nuclei visualization.

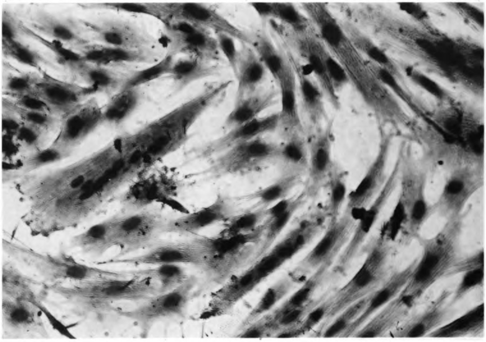


Figure 10. Cells placed in a 10^{-6} M insulin + 10^{-5} M ara-c serum-free medium, after 72 h of incubation.

Discussion

The growth data suggest that M2 porcine satellite cells do proliferate in culture, and the estimated 8.9pg DNA per cell is similar to the value previously reported by Doumit (1994).

These cells are also capable of undergoing differentiation, as indicated by creatine kinase induction, upon exposure to serum-free medium. Insulin- or insulin plus ara-c-stimulated creatine kinase activity (units/mg protein) were similar and numerically higher than the ara-c-stimulated creatine activity which is due to the inhibitory action of ara-c on protein synthesis, which may include creatine kinase protein and DNA synthesis (Turo & Florini, 1982).

Figure 11. Cells placed in a 10^{-5} M Ara-c only serum-free medium, after 72 h of incubation.

CHAPTER IV: BIOCHEMICAL EVIDENCE FOR THE PRESENCE OF βAR IN PORCINE SATELLITE CELL AND C2C12 CELL MEMBRANE PREPARATIONS

Abstract

C2C12 cell membrane preparations were incubated at 37°C for 2 h with increasing concentrations of [^{3}H]CGP12177 (specific activity 42.5 Ci/mmole) in the presence or absence of a 1000-fold concentration of unlabeled CGP12177. The specific binding of [^{3}H]CGP12177 to C2C12 cell membrane preparations was saturable, reversible, and of high affinity (K_D = 0.2 nM). The binding maximum (B_{max}) value was calculated to be 150 fmole/mg protein, which is similar to the B_{max} values previously reported by other investigators for both myocyte or adipocyte membrane preparations. The non-specific binding of [^{3}H]CGP12177 to C2C12 membrane preparations was approximately 10%. In other experiments, incubation of differentiated and non-differentiated porcine satellite cell membrane preparations with increasing concentrations of [^{3}H]CGP12177 produced a linear response, but unsaturable [^{3}H]CGP12177 specific binding was observed regardless of the protein concentration used (20-50 μg protein/mL) for non-differentiated porcine satellite cells. Little or no βAR presence was observed in differentiated cells.

Introduction

βAA are organic molecules, structurally similar to the mammalian neurotransmitter epinephrine. They are ligands of the guanine nucleotide-binding protein coupled glycoprotein receptors which belong to the seven membrane spanning-receptor family. The G protein is, in turn, coupled to either an effector or an enzyme, such as adenylyl cyclase. βAR transverse the plasma membrane with a stretch of about 20 to 25 hydrophobic amino acids with extracellular N-termini involved in ligand binding (Suryanarayana & Kobilka, 1993) and cytoplasmic C-termini involved in receptor desensitization (Hausdorff et al., 1989) and $G_{\alpha s}$ activation (Okamoto et al., 1991).

Several βAA have been shown to promote lean tissue deposition while depressing fat deposition in several species (Anderson et al., 1990; Baker et al., 1984; Barak et al., 1992; Bergen et al., 1989; Ricks et al., 1984). The anabolic effects of βAA on skeletal muscle may (Choo, Horan, Little & Rothwell, 1992) or may not (Smith, 1989) be βAR-mediated. Cook et al. (1994) reported that ractopamine enhanced the PDGF-stimulated proliferative activity of porcine satellite cells and thus concluded the presence of βAR in these cells even though βAR presence was not studied directly. Satellite cells play a significant role in muscle fiber growth (Allen et al., 1979) and muscle regeneration during injuries (Mauro, 1961).

Several models have been developed to study the mode of action of βAA in adipocytes but only a few for myocytes. Because myogenic C2C12 cells have been used to study other aspects of cell biology, the objectives of these studies were: (a) to determine if C2C12 cells could be used as a model to study the action of βAA, (b) to study the ontogeny of porcine satellite cell βAR, and (c) to utilize a hydrophilic, non-selective beta-antagonist to investigate the presence or absence of βAR in porcine satellite cells (just as researchers have used data derived from radiologand binding studies to establish the presence of βAR [Lacasa et al., 1986; Mersmann & McNeal, 1992]).

Experimental Procedures

Materials and Methods

Antibiotic-Antimycotic (ABAM), gentamicin, Minimum Essential Medium (MEM), Dulbecco's Modified Eagle Medium (DMEM), and Fetal Bovine Serum (FBS) were purchased from Gibco BRL (Grand Island, NY). Tissue culture (75 mm^2 culture plates) were purchased from Corning Glass Works (Corning, NY). Bovine pancreas insulin (I-1882), bovine transferrin (T-8027), MCDB-110 Medium (M-6520), dexamethasone (D-8893), bovine serum albumin (BSA, RIA grade, A-7888), water-soluble linoleic acid (L-5900), porcine skin gelatin (G-1890) were obtained from Sigma Chemical Company (St. Louis, MO). [^{3}H]CGP12177, specific activity 42.5 Ci/mmole, was purchased from DuPont (NEN). Scintillation Cocktail (Sentiverse®, SX18-4), Whatman GF/F filters and scintillation vials were purchased from Fisher Scientific.

Porcine Satellite Culture

Fifth passage porcine satellite cells were suspended in MEM containing 10% FBS, 0.5% ABAM, and 0.1% gentamicin. Cells were plated at a density of 300,000 cells/75 mm^2 flask previously coated with 0.1% gelatin as described by Richler and Yaffe (1970), and propagated in a humidified CO_2 incubator containing 95% air and 5% CO_2 at 37°C. Fresh medium was supplied every 24 h. For the non-differentiated porcine satellite cell radioligand binding assay, cells were harvested at the log phase of growth (3 to 5 d) for membrane preparation. For the differentiated porcine satellite cell radioligand binding assay, cells were allowed to become confluent (approximately 6 d). Differentiation was induced by switching cells to a serum-free medium as described by Merkel et al. (1993), except bFGF and PDGF-BB were not added, and 10^{-10} M dexamethasone was used, for 72 h before returning cells to MEM containing 10% FBS for 24 h to support completion of the fusion process. Cells were then harvested for membrane preparation.

C2C12 Cell Culture

C2C12 cells were suspended in DMEM containing 10% FBS, 0.5% ABAM, and 0.1% gentamicin, and plated at a density of 300,000 cells/75 mm^2 flask and grown in a humidified CO_2 incubator containing 95% air and 5% CO_2 at 37°C. Fresh medium was supplied every 48 h. Cells attained confluence at about 4 d, and differentiation was induced by switching cells to DMEM containing 2% FBS and 10^{-6} M insulin (differentiation medium) for 72 h before returning cells to the growth medium (DMEM + 10% FBS) for 24 h. Cells were then harvested for membrane preparation.

Membrane Preparation

Cell membranes were essentially prepared as described by Hausdorff et al. (1989). Medium was aspirated from monolayers at appropriate times (see culture conditions above) except cells were lysed with a polytron homogenizer at maximum setting. Cells were washed three times with ice-cold PBS, flasks were immediately placed on ice, and contents were scraped off into a 30 mL test tube containing 10 mL of a 5 mM Tris (pH 7.4)-2 mM EDTA buffer, and cells were lysed with a polytron homogenizer (four bursts for 5 s at maximum setting) on ice. The lysate was centrifuged at 200 x g for 20 min at 4°C to remove organelles and unbroken cells, and the supernatant was centrifuged at 40,000 x g for 20 min at 4°C. The resulting pellet was washed once with 5 mM Tris (pH 7.4)-2 mM EDTA buffer, and membrane pellets were rinsed and resuspended in a buffer containing 10 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 2 mM $CaCl_2$, and 10% glycerol. Aliquot samples were taken for protein content determination according to Bradford (1976). The remaining membrane suspension was diluted to 80 μg/mL with 10 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, and 10% glycerol buffer, and rapidly frozen in liquid nitrogen and maintained at -80°C for use within one week.

Radioligand Binding Assay

Radioligand binding assays were conducted according to Boege et al. (1988) using the hydrophilic non-selective beta-adrenergic antagonist [^{3}H]CGP12177 modified as follows: 40 μg/mL membrane protein, 0.45-15.6 nM [^{3}H]CGP12177, and incubation time of 2 h were used. Briefly, 40μg/mL membrane protein was incubated at 30°C for 2 h in a shaking water bath with increasing concentrations of [^{3}H]CGP12177 (specific activity 42.5 Ci/mmole) with or without a 1000-fold concentration of unlabeled CGP12177 in a 200 μL final volume. The incubations were stopped by the addition of 5 mL ice-cold PBS (pH 7.4), followed by rapid vacuum filtration of the suspension through GF/F glass fiber filters. The filters were washed twice with 5 mL ice-cold PBS (pH 7.4) and placed in scintillation vials containing 10 mL scintillation cocktail. After gentle shaking for 20 min, the vials were counted for ^{3}H activity with a scintillation counter at 40% efficiency.

Results and Discussion

The ontogeny of the porcine satellite cell βAR was investigated using membrane preparations from differentiated and non-differentiated porcine satellite cells. The results obtained from the binding of [^{3}H]CGP12177 to βAR in non-differentiated porcine satellite membrane preparations of 50, 40 and 20 μg membrane protein/mL are presented in Figure 12. Specific binding of [^{3}H]CGP12177 to non-differentiated porcine satellite cells was reversible and linear with the membrane protein concentration of 20 to 50 μg protein/mL. Non-specific binding, defined by the binding observed in the presence of a 1000-fold concentration (15.6 μM) of unlabeled CGP12177, was approximately 10%, which is consistent with the non-specific binding reported for this ligand in previous studies (Lacasa et al., 1986; Mersmann & McNeal, 1992). The specific binding was 90% as determined by delineation of total and non-specific bindings, but was not saturable at the highest ligand concentration (15.6 nM) studied.

A 60% reduction in membrane protein concentration still did not result in saturable specific binding. While some investigators have reported a range of

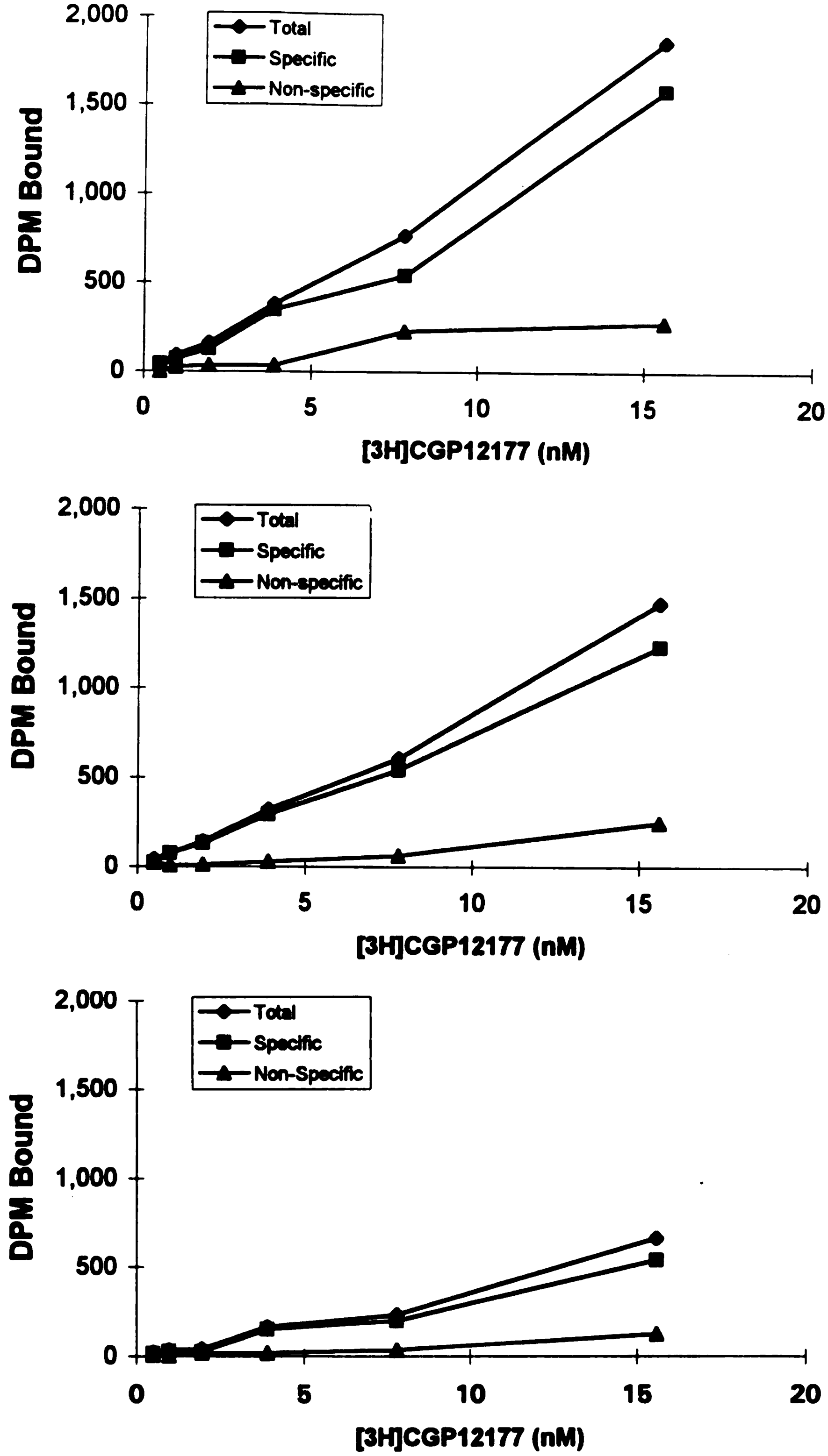


Figure 12. [^{3}H]CGP12177 binding to βAR in non-differentiated porcine satellite membrane preparations of 50, 40, and 20 μg membrane protein/mL, respectively.

<u>Figure 12</u>. [^{3}H]CGP12177 binding to βAR in non-differentiated porcine satellite membrane preparations of 50, 40, and 20 μg membrane protein/mL, respectively.

<u>Note</u>. Fifty, 40, and 20 μg membrane protein/mL, respectively, harvested from non-differentiated porcine satellite cells, were incubated with increasing concentrations of [^{3}H]CGP12177 with or without a 1000-fold concentration of unlabeled CGP12177 at 30°C for 2 h. Each data point represents a mean of duplicate incubations.

0.2-5 nM K_D values for βAR, others have also suggested higher K_D values for βAR. Green et al. (1993) reported a K_i value of 368 $\pm$ 89 nM for human β_2-AR. Thus, a much higher ligand ([^{3}H]CGP12177) concentration may have been required to attain saturation of βAR in non-differentiated cells used here.

The binding characteristics of [^{3}H]CGP12177 to differentiated porcine satellite cell membrane at 30°C is depicted in Figure 13. Specific binding was very close to background at a concentration range of 0.49-1.95 nM [^{3}H]CGP12177. At higher [^{3}H]CGP12177 concentrations, non-specific binding, as defined by the binding observed in the presence of 15.6 nM unlabeled CGP12177, equaled total binding, indicating little or no βAR presence. The absence of a noticeable binding observed in differentiated porcine satellite cell membrane preparation may have been due to two reasons. First, the differentiation milieu--serum-free medium-- may have been too harsh to sustain viable porcine satellite cells for the 4 d required to complete the differentiation process. The serum-free medium lacks growth factor that may have been required for the expression of the βAR gene. On equal cell number basis, non-differentiated porcine satellite cells produced three-fold more total cellular protein than differentiated cells. Of course, it is tempting to speculate that a three-fold less βAR protein will be produced if the individual protein constituting the total cellular proteins are equally sensitive to serum starvation. However, the possibility that some proteins may be more sensitive to serum deprivation than others also exists. Secondly, an age-associated βAR modification, or differential gene expression, that may impair its affinity for the ligand used in these studies also remains a possible explanation for the lack of βAR activity observed in differentiated porcine satellite cell membrane preparation. For example, substitution of a single amino acid in the purported ligand binding pocket of human β_2-AR may either decrease the affinity by approximately four-fold (1450 $\pm$ 79 versus 368 $\pm$ 39 nM) (Green et al., 1993) or allow βAR to recognize an antagonist as agonist (Suryanarayana & Kobilka, 1993).

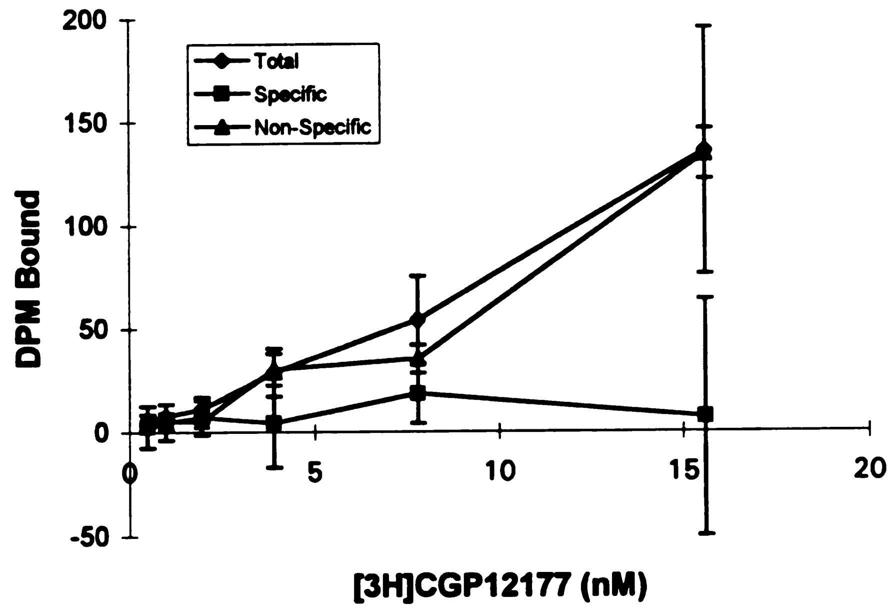


Figure 13. [^{3}H]CGP12177 binding to βAR in differentiated porcine satellite cell membrane preparation.

Note. 50 μg membrane protein/mL harvested from differentiated porcine satellite cells were incubated, with increasing concentrations of [^{3}H]CGP12177 with or without a 1000-fold concentration of unlabeled CGP12177 at 30°C for 2 h. Cells were differentiated as described under the Materials and Methods section. Each data point represents a mean of three experiments done in duplicate.

[^{3}H]CGP12177 binding to βAR in differentiated C2C12 cell membrane preparation was studied and results obtained by incubating 40 μg membrane protein/mL with increasing concentrations of [^{3}H]CGP12177 (0.49-15.6 nM) in the presence or absence of 15.6 μM unlabeled CGP12177 are presented in Figure 14.

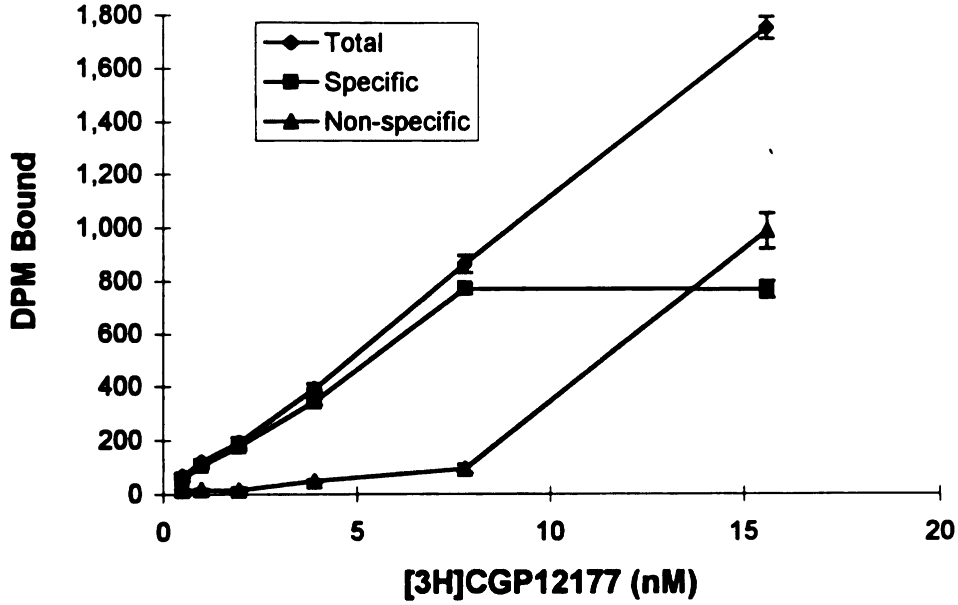


Figure 14. [^{3}H]CGP12177 binding to βAR in C2C12 cell membrane preparation.

Note. 40 μg membrane protein/mL harvested from differentiated C2C12 cell was incubated with increasing concentrations of [^{3}H]CGP12177, with or without a 1000-fold concentration of unlabeled [^{3}H]CGP12177 at 30°C for 2 h. C_2C_{12} cells were differentiated as described under the Materials and Methods section. Each data point represents a mean of three experiments done in duplicate.

The specific binding was saturable, reversible, and of high affinity (K_D = 0.2 nM) as determined by the Scatchard Plot. Scatchard plot for specific binding was linear and fit a correlation coefficient of >.96 (see Figure 15). The model showed no evidence for curvilinearity to suggest a two-affinity site receptor. The non-specific binding was approximately 10% which is consistent with the 10-15% previously reported for [^{3}H]CGP12177 by Lacasa et al. (1986) and 10% reported by Mersmann and McNeal (1992). The literature suggests that the K_D for βAR is ligand- and cell-type-dependent. For example, using a ligand [^{3}H]CGP12177 in human adipocyte membranes, the K_D value was reported to be 0.9 nM (Mauriege et al., 1988), whereas with the

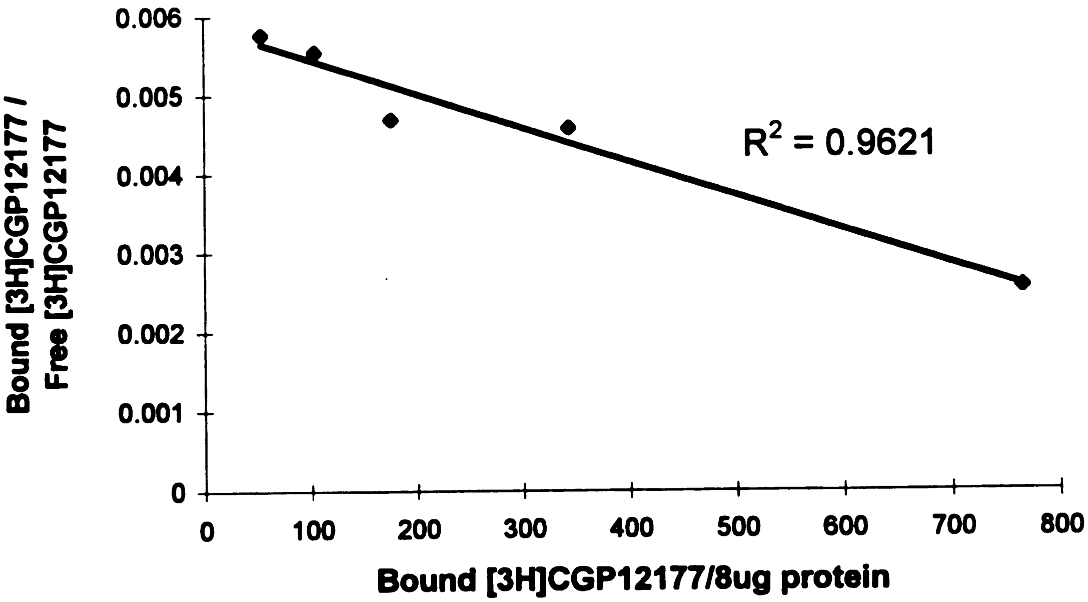


Figure 15. Scatchard Plot of specifically bound [^{3}H]CGP12177 to the differentiated C2C12 cell membrane preparation.

Note. C2C12 cells were grown and differentiated as described under the Materials and Methods section. Each data point represents the mean of three experiments done in duplicate.

same ligand [^{3}H]CGP12177 in pig adipocyte crude membrane preparation, a K_D value of 0.6 nM was reported (Mersmann & McNeal, 1988). Lacasa et al. (1986) suggested a K_D value of 1.2 ± .3 using intact human adipocyte. In the present studies, the B_{max} was calculated to be 150 fmole/mg protein, which is similar to the B_{max} values indicated in previous studies. Mersmann and McNeal (1992) reported a B_{max} of >150 fmole/mg protein. Coutinho et al. (1990) reported 170 fmole/mg protein. The K_D (0.2 nM) and B_{max} (150 fmole/mg protein) values reported in the present studies are characteristic of typical βAR. Thus, we are reporting here the presence of typical βAR in C2C12 cell membrane preparation.

CHAPTER V: BETA-ADRENERGIC AGONISTS-STIMULATED cAMP ACCUMULATION IN PORCINE SATELLITE AND C2C12 CELLS

Abstract

Two experiments were conducted to measure ISO-, RAC-, and CLEN-stimulated cAMP accumulation in both differentiated and non-differentiated porcine satellite cells, and differentiated C2C12 cells.

In Experiment 1, post-differentiated C2C12 cells were washed three times and overlaid with 5 mL PBS (pH 7.4) containing either ISO, RAC, or CLEN ($10^{-9, -7, -5}$ M). Forskolin was added at 10^{-5} M as a positive control; for negative control, no drug was added. Ten μM cAMP phosphodiesterase inhibitor was added to each incubation to prevent the conversion of cAMP to AMP. Results indicate that all concentrations of the βAA ISO, RAC, and CLEN studied resulted in cAMP release higher than the negative control. At $10^{-7, -5}$ M, CLEN, ISO, and 10^{-5} RAC equaled or exceeded the Forskolin-induced cAMP release per unit of cellular protein. Release of cAMP was increased ($P < .01$) by 10^{-5} M ISO, RAC, and CLEN; however, the RAC response was somewhat less than ISO or CLEN.

In Experiment 2, either differentiated or non-differentiated porcine satellite cells were washed three times and overlaid with 5 mL PBS (pH 7.4) containing either ISO, RAC, or CLEN ($10^{-9, -7, -5}$ M). Forskolin was added at 10^{-5} M as a positive control; for negative control, no drug was added. Results showed that at 10^{-5} M, ISO

significantly increased cAMP release ($P < .001$) in differentiated but not in non-differentiated porcine satellite cells. The Forskolin-induced cAMP release was significantly higher ($P < .001$) compared to negative controls in both differentiated and non-differentiated porcine satellite cells. These results suggest the the presence of functional βAR-Adenylyl Cyclase systems in differentiated and non-differentiated porcine satellite cells and differentiated C2C12 cells.

Introduction

Beta-Adrenergic Receptors (βAR) belong to the Seven Membrane Spanning-receptor family. Sutherland and Rall (1960) reported that βAR are coupled to the enzyme adenylyl cyclase, an enzyme that utilizes ATP and generates cAMP as a product. PKA is activated by cAMP association with its regulatory subunits, thus allowing the catalytic subunits to dissociate from the regulatory subunits to either activate or deactivate a series of intracellular enzymes by phosphorylation. Thus, the binding of a βAA to its receptor, βAR, results in the activation of PKA which evokes a pleiotropic effect on the metabolic processes in animals, including meat-producing animals.

The lipolytic and antilipogenic effects of βAA on adipose tissue (Peterla & Scanes, 1990; Weber et al., 1992) and lean tissue-enhancement effect (Anderson et al., 1990; Bergen et al., 1989; Ricks et al., 1984) have been reported. However, the mechanism of action of βAA is a matter of controversy; βAA actions may (Choo et al., 1992) or may not (Smith, 1989) be mediated by βAR in muscle tissue.

At the present time, the preponderance of evidence for functional βAR-adenylyl cyclase systems in meat animal research has emanated from studies that evaluated the effects of βAA on NEFA and glycerol release, and FAS, ACC, and malic enzyme (ME) activities. The present studies seek to investigate the effect of ISO, RAC, and CLEN binding to βAR in porcine satellite cells and C2C12 cells grown in culture, and the subsequent biological consequence--a change in the cAMP concentration.

Experimental Procedures

Materials and Methods

ABAM, gentamicin, MEM, DMEM, and FBS were purchased from Gibco BRL (Grand Island, NY). Tissue culture plates (100 x 20 mm) were purchased from Corning Glass Works (Corning, NY). Bovine pancreas insulin (I-1882), bovine transferrin (T-8027), MCDB-110 Medium (M-6520), dexamethasone (D-8893), BSA (RIA grade A-7888), water-soluble linoleic acid (L-5900), porcine skin gelatin (G-1890), cyclic AMP phosphodiesterase inhibitor (B-8279), isoproterenol hydrochloride (I-6504), and forskolin (F-6886) were purchased from Sigma Chemical Company (St. Louis, MO). Cyclic AMP kits ($KAPH_2$) were obtained from Diagnostic Products, Inc. (Los Angeles, CA).

Porcine Satellite Cell Growth and Differentiation

Fifth passage porcine satellite cells were suspended in MEM containing 10% FBS, 0.5% ABAM, and 0.1% gentamicin. Cells were plated at a density of 100,000 cells/100 mm diameter flask previously coated with 0.1% gelatin as described by Richler and Yaffe (1970), and propagated in a humidified CO_2 incubator containing 95% air and 5% CO_2 at 37°C. Fresh medium was supplied every 24 h. For the non-differentiated porcine satellite cell cAMP assay, cells were utilized at the log phase of growth (3 to 5 d). For the differentiated porcine satellite cell cAMP assay, cells were allowed to become confluent (approximately 6 d). Differentiation was induced by switching cells to a serum-free medium as described by Merkel et al. (1993), except bFGF and PDGF-BB were not added, and 10^{-10} M dexamethasone was used, for 72 h before returning cells to MEM containing 10% FBS for 24 h to support completion of the fusion process.

C2C12 Cell Growth and Differentiation

C2C12 cells were suspended in DMEM containing 10% FBS, 0.5% ABAM, and 0.1% gentamicin. Cells were plated at a density of 100,000 cells/100 mm diameter flask and grown in a humidified CO_2 incubator containing 95% air and 5% CO_2 at 37°C. Fresh medium was supplied every 48 h. Cells attained confluence at about 4 d, and differentiation was induced by switching cells to DMEM containing 2% FBS and 10^{-6} M insulin (differentiation medium) for 72 h before returning cells to the growth medium (DMEM + 10% FBS) for 24 h. Cells were then utilized for the cAMP assay.

βAA-Stimulated cAMP Accumulation

Medium was aspirated from monolayers (porcine satellite cells and C2C12 cells) and washed three times with PBS (pH 7.4). Monolayers were overlaid with 5 mL PBS (pH 7.4) previously incubated to 37°C in a water bath; either ISO, RAC, or CLEN was added at a final concentration of 10^{-5}, 10^{-7}, and 10^{-9} M. To other monolayers as positive control, forskolin was added at 10^{-5} M final concentration, while negative control was achieved by not adding any drugs. Ten μM cAMP phosphodiesterase inhibitor was added to each incubation to prevent enzymatic degradation of cAMP. Cells were incubated in a humidified CO_2 incubator containing 95% air and 5% CO_2 at 37°C for 5 min. Following the incubation period, a 100 μL aliquot sample/plate was removed and placed in a 12 x 75 mm polypropylene tube and placed in a 0°C ice bath. The cell monolayers and 100 μL aliquot samples were kept at -20°C for protein determination and cAMP analysis respectively (within 3 d). Total cellular proteins/plate were determined according to Bradford (1976). The extracellular cAMP analysis was performed using the Diagnostic Products Incorporated (DPC) cAMP kits per manufacturer's instructions. This procedure to measure cAMP is based on the principle of competitive protein binding by keeping the cAMP binding protein and ^{3}H cAMP constant, while adding increasing amounts of unlabeled cAMP, resulting in increasing displacement of ^{3}H cAMP by cAMP. Thus, the cAMP binding protein-^{3}H cAMP counts are obtained as a function of the unlabeled cAMP concentration and are plotted to generate a calibration curve from which unknown samples may be read.

Results and Discussion

Results from the βAA-induced cAMP studies in differentiated and non-differentiated porcine satellite cell studies are presented in Figures 16 and 17. Differentiated and non-differentiated porcine satellite cells responded to βAA stimulation; however, the magnitude of response of differentiated cells was greater. 10^{-5} M ISO significantly increased cAMP release ($P < .001$) in differentiated but not in non-differentiated porcine satellite cells. 10^{-5} M ISO numerically, but not significantly, increased cAMP release in non-differentiated cells. Forskolin-stimulated cAMP accumulation was significant ($P < .001$) in both differentiated and non-differentiated porcine satellite cells compared to the control. In these studies, 10^{-7} and 10^{-5} M RAC or CLEN marginally increased extracellular cAMP release in differentiated, but not in non-differentiated, porcine satellite cells, thus suggesting that RAC and CLEN may be

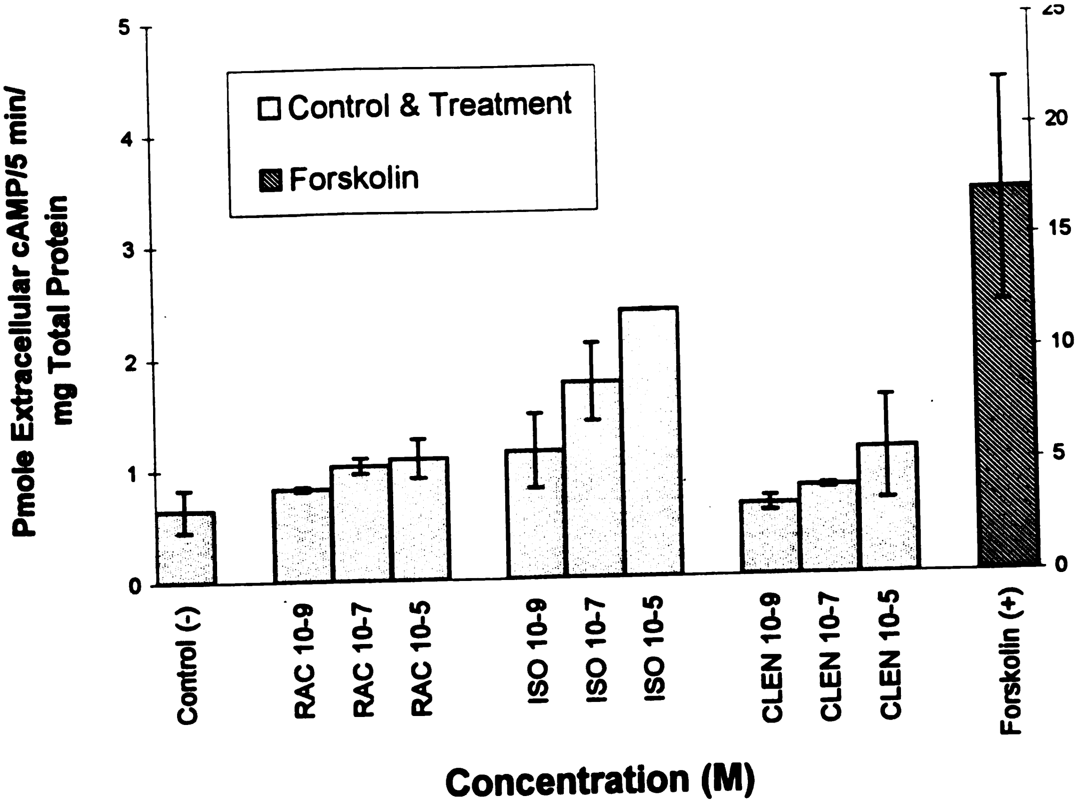


Figure 16. βAA-stimulated cAMP accumulation in differentiated porcine satellite cells.

Note. Porcine satellite cells were propagated and differentiated as described in the Materials and Methods section. Either ISO, RAC, or CLEN was added at a final concentration of 10^{-9}, 10^{-7}, or 10^{-5} M as a positive control. Forskolin was added at 10^{-5} M. Monolayers were then incubated at 37°C for 5 min and cAMP quantified as stated under the Materials and Methods section. Data are means ± SD for three experiments done in duplicate.

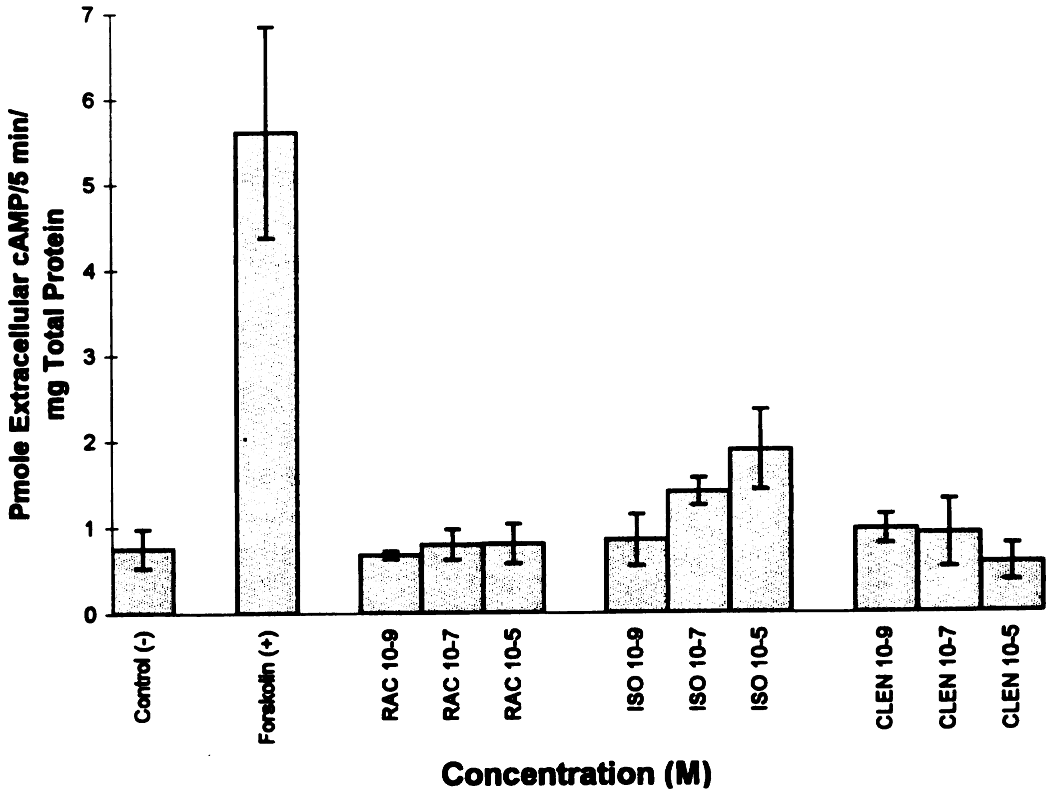


Figure 17. βAA-stimulated cAMP accumulation in non-differentiated porcine satellite cells.

Note. Porcine satellite cells were propagated and differentiated as described in the Materials and Methods section. Either ISO, RAC, or CLEN was added at a final concentration of 10^{-9}, 10^{-7}, or 10^{-5} M as a positive control. Forskolin was added at 10^{-5} M. Monolayers were then incubated at 37°C for 5 min and cAMP quantified as stated under the Materials and Methods section. Data are means $\pm$ SD for three experiments done in duplicate.

poorly coupled to the βAR-adenylyl cyclase systems at least in porcine satellite cells. These observations are consistent with previous reports. RAC or CLEN were effective inhibitors (antagonists) of epinephrine-stimulated lipolysis in pig adipocytes (Liu & Mills, 1989). In contrast, RAC or CLEN may also be a weak agonist (Coutinho et al., 1990) in pig adipocytes. Clenbuterol increased plasma concentration of free fatty acids, suggesting that agonist response occurs as well (Mersmann, 1987). These latter reports are corroborated by in vivo studies (Merkel et al., 1987). These findings not only indicate the presence of functional βAR-adenylyl cyclase systems in both cell types, but also suggest that either the differentiated cell βAR-adenylyl cyclase system may be more sensitive to agonist (ISO) activation, or that βAR may be coupled to a different species of G protein that is a stronger stimulator of adenylyl cyclase. The existence of multiple forms of G_{α}-stimulation ($G_{\alpha s}$) was reported (Jones & Reed, 1987).

Results from the C2C12 cell response to beta-adrenergic stimulation are presented in Figure 18. The control differentiated C2C12 incubations resulted in negligible cAMP release; forskolin-stimulated cAMP accumulation was 17-fold more than the controls. All concentrations of the βAA tested resulted in cAMP release higher than the controls; at $10^{-7,-5}$ M, CLEN, ISO, and 10^{-5} M RAC equaled or exceeded forskolin cAMP release per unit of cellular protein. Release of cAMP was increased ($P < .01$) by all β-adrenergic agonists at 10^{-5} M; RAC response was somewhat less than ISO or CLEN.

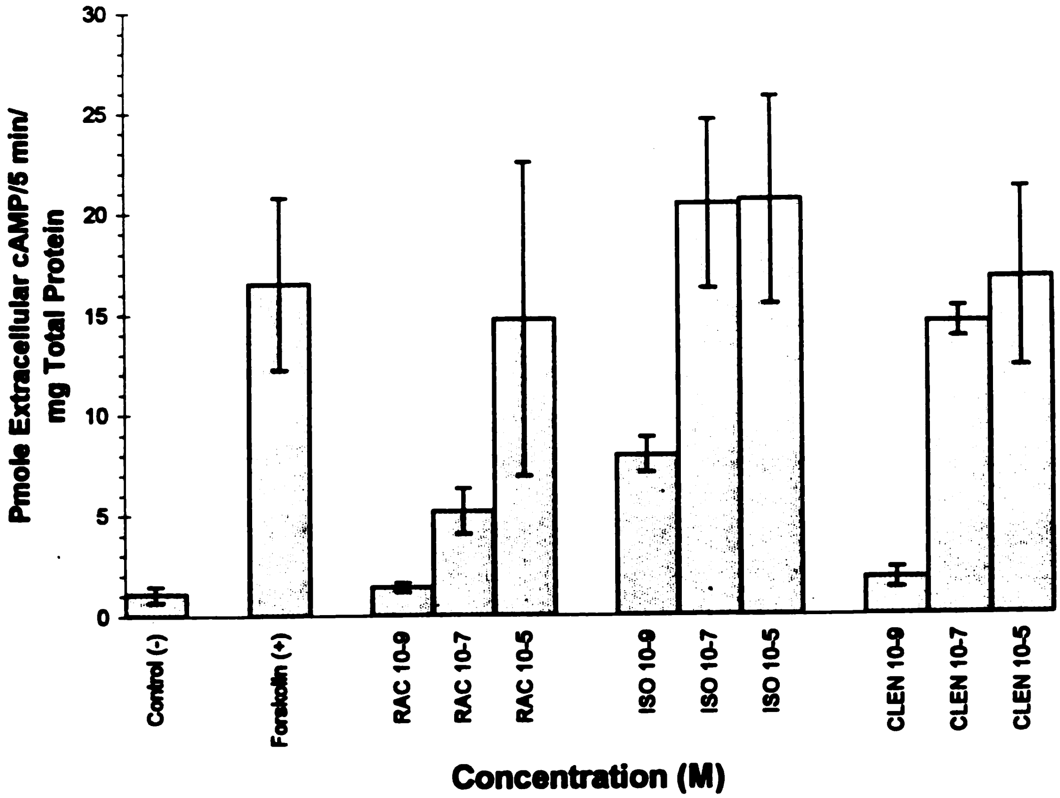


Figure 18. βAA-stimulated cAMP accumulation in differentiated C2C12 cells.

Note. C2C12 cells were grown and differentiated as described under the Materials and Methods section. Either ISO, RAC, or CLEN was added at a final concentration of 10^{-9}, 10^{-7}, or 10^{-5} M. Monolayers were then incubated at 37°C for 5 min and cAMP was quantified as stated under the Materials and Methods section. Data are means ± SD for three experiments done in duplicate.

CHAPTER VI: REVERSE TRANSCRIPTION AND POLYMERIZATION OF MESSENGER RNA IN PORCINE SATELLITE AND C2C12 CELLS

Introduction

Our biochemical and physiological studies have suggested the presence of functional βAR-Adenylyl Cyclase Systems in both porcine satellite and C2C12 cells, although the evidence is much stronger for C2C12 cells. However, because of the differences observed in both the binding of [^{3}H]CGP12177 to βAR, and cAMP response studies in differentiated versus non-differentiated cells, it became of interest to evaluate the level of βAR messenger RNA in the cell types of satellite cells by utilizing the reverse transcription-polymerase chain reaction (RT-PCR) technique.

Experimental Procedures

Materials and Methods

ABAM, gentamicin, MEM, DMEM, and FBS were purchased from Gibco BRL (Grand Island, NY). Tissue culture plates (75 cm^2/flask) were purchased from Corning Glass Works (Corning, NY). Bovine pancreas insulin (I-1882), bovine transferrin (T-8027), MCDB-110 medium (M-6520), dexamethasone (D-8893), BSA (RIA grade A-7888), water-soluble linoleic acid (L-5900), and porcine skin gelatin (G-1890) were purchased from Sigma Chemical Company (St. Louis, MO). RNase inhibitor and DNA molecular weight marker V were obtained from Boehringer Mannheim. RT-PCR Kit (N808-0179) and PCR reaction micro tubes were purchased from Perkin Elmer. Total RNA/mRNA isolation reagent (RNA STAT-60) was obtained from TEL-TEST "B," Inc., Friendswood, TX.

Porcine Satellite Cell Growth and Differentiation

Fifth passage porcine satellite cells were suspended in MEM containing 10% FBS, 0.5% ABAM, and 0.1% gentamicin, plated at a density of 300,000 cells/75 mm^2 flask previously coated with 0.1% gelatin as described by Richler and Yaffe (1970), and propagated in a humidified CO_2 incubator containing 95% air and 5% CO_2 at 37°C. Fresh medium was supplied every 24 h. For the non-differentiated porcine satellite cell RT-PCR assay, cells were harvested at the log phase (3 to 5 d) of growth for total RNA isolation. For the differentiated porcine satellite cell RT-PCR assay, cells were allowed to become confluent (approximately 6 d). Differentiation was induced by placing cells in a serum-free medium as described by Merkel et al. (1993), except bFGF and PDGF-BB were not added, and dexamethasone used at 10^{-10} M, for 72 h before returning cells to MEM containing 10% FBS for 24 h to support completion of the fusion process. Total RNA was isolated from the cells using the RNA STAT-60 Kit, which includes phenol and guanidinium thiocyanate in a monophase solution, per manufacturer's protocol (described under the Total RNA Isolation section).

C2C12 Cell Growth and Differentiation

C2C12 cells were suspended in DMEM containing 10% FBS, 0.5% ABAM, and 0.1% gentamicin, plated at a density of 300,000 cells/75 mm^2 flask and grown in a humidified CO_2 incubator containing 95% air and 5% CO_2 at 37°C. Fresh medium was supplied every 48 h. Cells attained confluence at about 4 d, and differentiation was induced by switching cells to DMEM containing 2% FBS and 10^{-6} M insulin (differentiation medium) for 72 h before returning cells to the growth medium (DMEM + 10% FBS). Total RNA was isolated from the cells at times indicated under the Materials and Methods section.

Total RNA Isolation and Treatment

Homogenization. Medium was aspirated from cell monolayers, 6 mL RNA STAT reagent/75 cm^2 flask was added in a fume hood, and cells were lysed by repetitive pipetting. Following homogenization, cells were stored at room temperature for 5 min to permit the complete dissociation of nucleoprotein complexes. The Homogenates were transferred from 75cm^2 culture flasks into 30 ml Corex glass tubes.

Extraction. 1.2 mL chloroform was added to each tube. The tubes were covered tightly and shaken vigorously for 15 s. After 3 min at room temperature, the homogenates were centrifuged at 12,000 g for 15 min at 4° C. Following centrifugation, the homogenates separated into two phases: a lower red phenol chloroform phase containing DNA and proteins, and a colorless upper aqueous phase containing RNA.

Precipitation. The aqueous phase (approximately 3.5 mL) was removed from each 30 mL Corex tube and transferred to a fresh 30 mL Corex tube, and 3 mL isopropanol was added to each tube. Samples were stored at room temperature for 5-10 min before centrifugation at 12,000 g for 10 min at 4° C. The supernatant was removed from each tube.

Wash. The remaining RNA pellets were washed with 6 mL 75% cold ethanol/tube by vortexing and were subsequently centrifuged at 7,500 g for 5 min at 4° C to recover the RNA pellets. Pellets were resuspended in Diethyl/Pyrocarbonate (DEPL)-treated water. Aliquot RNA samples were taken from each cell type and analyzed by spectrophotometer and electrophoresis gel. The remaining RNA samples were diluted with DEPC-treated water to 0.5 $\mu g/\mu L$ and stored at -80° C for use within 1 wk.

Prior to use for reverse-transcription-polymerase chain reaction (RT-PCR), aliquots of RNA samples from all cell types (porcine satellite and C2C12 cells) were DNase 1-treated and incubated for 30 min at 37° C to degrade all possible contaminating DNA. Following incubation, the residual DNase activity was stopped by the addition of 20 mM EDTA, a chelating agent, to deactivate DNase 1. The RNA samples were further treated with RNase inhibitor (40 units/μL stock) to deactivate all contaminating RNases, if any.

Oligonucleotides

Porcine satellite and C2C12 cell RNA were probed for all subtypes of βAR. The following 21-mer primers presented in Table 3 were used in the primer-directed DNA polymerization. These 21-mers were made at the Michigan State University Macromolecule and Structural Facility.

Table 3. The oligonucleotide sequences used as primer for the three sub-types of βAR.

Primer	Receptor	Strand	Sequence (5'→3')	Predicted Product Size (bp)
A	β_3-AR	Sense	GAG ACT CCA GAC CAT GAC CAA	325
		Anti-sense	TCA TGA TGG GGG CAA ACG ACA	
B	β_3-AR	Sense	TGT CGT TTG CGC CCA TCA TGA	534
	β_3-AR	Anti-sense	TAG AGT ACC GGG TTG AAG GCA	
C	β_3-AR	Sense	TGT CGT TTG CGC CCA TCA TGA	415
	β_3-AR	Anti-sense	CAA CCA GCA GAG ACT GAA GGT	
D	β_2-AR	Sense	GCA GAC GGT CAC CAA CTA CTT	464
		Anti-sense	ACG AAG ACC ATG ATC ACC AGG	
E	β_2-AR	Sense	CCT GCT GAT CAT GGT CTT CGT	372
		Anti-sense	GGC AAT CCT GAA ATC TGG GCT	
F	β_1-AR	Sense	ACG CTC ACC AAC CTC TTC ATC	463
		Anti-sense	TAC AGG AAG GCC ATG ATG CAC	
G	β_1-AR	Sense	GTG CAT CAT GGC CTT CGT GTA	376
	β_1-AR	Anti-sense	TGA AGA AGA CGA AGA GCC CCT	

Figure 19 shows a computer-generated line-up file indicating regions of homology between human and bovine βAR where primer selections were made. The conserved sequences between human and bovine β_3-AR are boxed. No conserved regions were found between human and bovine β_1-AR and β_2-AR. Therefore, primers for β_1-AR and β_2-AR were made against areas of low GC contents in human and bovine βAR 1 and 2, hoping these regions are conserved in porcine. Each primer pair (sense and antisense) was targeted to amplify specific regions of the cDNA derived from the reverse transcriptase reaction in primer-directed DNA polymerization reaction.

Because only the targeted regions of the DNA must be amplified, two primers (sense and antisense) were required. For example, for primer A, β_3-AR, the expected RT-PCR product was 325 base pair (bp). The sense primers were designed to anneal at base 271 to 291, and extended from one direction, while its antisense primer annealed at base 596-576, and extended from the opposite direction to meet at a point of termination. Thus base 271 to 596 was amplified, resulting in a RT-PCR product of 325 bp.

```
 bovbeta123.msf  MSF: 1447  Type: N  March 10, 1996 18:05  Check: 599 ..

 Name: bovbeta3        Len:  1291  Check: 6510  Weight:  1.00
 Name: humbeta3        Len:  1280  Check: 4841  Weight:  1.00
 Name: bovbeta2        Len:   447  Check: 2162  Weight:  1.00
 Name: humbeta2        Len:  1331  Check: 7721  Weight:  1.00
 Name: humbeta1        Len:  1447  Check: 6466  Weight:  1.00
 Name: bovbeta123      Len:  1447  Check: 2899  Weight:  1.00

//

             1                                                  50
   bovbeta3  .......... .......... .......... .......... ..........
   humbeta3  .......... .......... .......... .......... ..........
   bovbeta2  .......... .......... .......... .......... ..........
   humbeta2  .......... .......... .......... .......... ..........
   humbeta1  ATGGGCGCGG GGGTGCTCGT CCTGGGCGCC TCCGAGCCCG GTAACCTGTC
 bovbeta123  ATGGGCGCGG GGGTGCTCGT CCTGGGCGCC TCCGAGCCCG GTAACCTGTC

             51                                                100
   bovbeta3  .......... .........A TGGCTCCGTG GCCTCCTGGG AACAGCTCTC
   humbeta3  .......... ........A. TGGCTCCGTG GCCTCACGAG AACAGCTCTC
   bovbeta2  .......... .......... ..TTTCTCCT CCCCCAGGTG ATATCCACTC
   humbeta2  .......... .......... .......... ...ATGGGGC AACCCGGGAA
   humbeta1  GTCGGCCGCA CCGCTCCC.. CG........ ACGGCGCGGC CACCGCGGCG
 bovbeta123  GTCGGCCGCA CCGCTCCC.. tGgctccgtg gCctc..Ggg aac.gc.ctc
```

Figure 19. Region of homology between human and bovine βAR.

```
             101                                                150
   bovbeta3  TGACCCCGTG GCCAGATATC CCCACCCTGG CACCCAATAC TGCCAACGCG
   humbeta3  TTGCCCCATG GCCGGACCTC CCCACCCTGG CGCCCAATAC CGCCAACACC
   bovbeta2  TGTTCCCCTG TGTAGTCAGT CCTGTCATTG CTGTTGCTGG AGCAGCCATT
   humbeta2  CGGCAGCGCC TTCTTGC..T GGCACCCAAT AGAAGCCATG CGCCGGACCA
   humbeta1  CGGCTGCTGG TGCCCGCGTC GCCGCCCGCC TCGTTGCTGC CTCCCGCCAG
 bovbeta123  tggccccgtg t.cag.catc cccacCctgg cg....ct.c cgCc..c.cg

             151                                                200
   bovbeta3  AGTGGGCTGC CAG....GGG TGCCCTGGGC GGTGGCGCTG GCGGGGGCGC
   humbeta3  AGTGGGCTGC CAG....GGG TTCCGTGGGA GGCGGCCCTA GCCGGGGCCC
   bovbeta2  CCCATAGGCC TTCAATGAAG ACCTGCGCAG GCAGAGAAGC TCCTGGAAGG
   humbeta2  CGACGTCACG CAGCAAAGGG ACGAGGTGTG GGTGGTGGGC ATGGGCATCG
   humbeta1  CGAAAGCCCC GAGCCGCTGT CTCAGCAGTG GACAGCGGGC ATGGGTCTGC
 bovbeta123  cg..ggctcc cag....ggg ..c.g.gg.g Gg.ggcg.gc .cggGg..gc

             201                                                250
   bovbeta3  TGTTGGCGCT AGCGGTGCTG GCCACCGTGG GAGGCAACCT GCTGGTAATC
   humbeta3  TGCTGGCGCT GGCGGTGCTG GCCACCGTGG GAGGCAACCT GCTGGTCATC
   bovbeta2  CAATCCTGAA ATCTGGGCTC CGGCAGTAGA TAAGGGGATT GAAAGCGGAG
   humbeta2  TCATGTCTCT CATCGTCCTG GCCATCGTGT TTGGCAATGT GCTGGTCATC
   humbeta1  TGATGGCGCT CATCGTGCTG CTCATCGTGG CGGGCAATGT GCTGGTGATC
 bovbeta123  tgaTggcgct ..c.GtgCTg gcca.cgtGg .agGcaa..T GctgGt.atc
```

β_3-AR (primer A Sense)
(271-291)

```
             251                                                300
   bovbeta3  GTGGCCATCG CCCGGACGCC GAGACTCCAG ACCATGACCA ACGTGTTCGT
   humbeta3  GTGGCCATCG CCTGGACTCC GAGACTCCAG ACCATGACCA ACGTGTTCGT
   bovbeta2  TTGATGTAGC CCAACCAGTT TAGAAGGATG TATATTTCCT TACGGATGAG
   humbeta2  ACAGCCATTG CCAAGTTCGA GCGTCTGCAG ACGGTCACCA ACTACTTCAT
   humbeta1  GTGGCCATCG CCAAGACGCC GCGGCTGCAG ACGCTCACCA ACCTCTTCAT
 bovbeta123  gtggccatcg CCaagacgcc gaGactgcaG ac.aT.aCCa ac.tgtTcat
```

β_2-AR (primer D Sense)
(277-297)

β_1-AR (primer F Sense)
(281-301)

```
             301                                                350
   bovbeta3  GACTTCGCTG GCCACAGCCG ACCTGGTGGT GGGGCTCCTG GTCGTGCCCC
   humbeta3  GACTTCGCTG GCCGCAGCCG ACCTGGTGAT GGGACTCCTG GTGGTGCCGC
   bovbeta2  GTTATCCTTG ATCACGTGCA CAATGTTGAC AATGAAGAAG GGCAGCCAGC
   humbeta2  CACTTCACTG GCCTGTGCTG ATCTGGTCAT GGGCCTGGCA GTGGTGCCCT
   humbeta1  CATGTCCCTG GCCAGCGCCG ACCTGGTCAT GGGGCTGCTG GTGGTGCCGT
 bovbeta123  gactTC.cTG gcCacagccg accTGgTgat ggggctgctg GtggtgCcgc

             351                                                400
   bovbeta3  CGGGGGCCAC GTTGGCGCTG ACCGGCCACT GGCCCCTGGG CGTCACCGGT
   humbeta3  CGGCGGCCAC CTTGGCGCTG ACTGGCCACT GGCCGTTGGG CGCCACTGGC
   bovbeta2  ACAGGGTGAA AGTGCCCATG ATAATGCCTA AAGTCTTGGG GGCTTTGTGT
   humbeta2  TTGGGGCCGC CCATATTCTT ATGAAAATGT GGACTTTTGG CAACTTCTGG
   humbeta1  TCGGGGCCAC CATCGTGGTG TGGGGCCGCT GGGAGTACGG CTCCTTCTTC
 bovbeta123  ..ggGGccac cttggcgcTg a.gggccact gg.c.ttgGG cgccttctg.
```

<u>Figure 19.</u> (cont'd).

```
            401                                              450
bovbeta3    TGCGAGCTGT GGACCTCAGT GGACGTGCTG TGTGTGACCG CCAGCATCGA
humbeta3    TGCGAGCTGT GGACCTCGGT GGACGTGCTG TGTGTGACCG CCAGCATCGA
bovbeta2    TCCTTCAAGT AGAACTTGGA GGTCCTGCGT TGTCCTAGAC CCGTCCG
humbeta2    TGCGAGTTTT GGACTTCCAT TGATGTGCTG TGCGTCACGG CCAGCATTGA
humbeta1    TGCGAGCTGT GGACCTCAGT GGACGTGCTG TGCGTGACGG CCAGCATCGA
bovbeta123  TgCgagctgT gGAccTc.gt gGacgTGCtg TGtgtgAc.g CCagCatcGA

            451                                              500
bovbeta3    AACCCTGTGC GCCCTGGCGG TGGACCGCTA CCTGGCCGTG ACCAACCCGC
humbeta3    AACCCTGTGC GCCCTGGCCG TGGACCGCTA CCTGGCTGTG ACCAACCCGC
humbeta2    GACCCTGTGC GTGATCGCAG TGGATCGCTA CTTTGCCATT ACTTCACCTT
humbeta1    GACCCTGTGT GTCATTGCCC TGGACCGCTA CCTCGCCATC ACCTCGCCCT
bovbeta123  .ACCCTGTGc G.c.TgGCcg TGGAcCGCTA CcTgGCc.Tg ACc..cCCg.

            501                                              550
bovbeta3    TGCGCTACGG CGCGCTGGTC ACCAAACGCC GCGCCCTAGC AGCCGTGGTC
humbeta3    TGCGTTACGG CGCACTGGTC ACCAAGCGCT GCGCCCGGAC AGCTGTGGTC
humbeta2    TCAAGTACCA GAGCCTGCTG ACCAAGAATA AGGCCCGGGT GATCATTCTG
humbeta1    TCCGCTACCA GAGCCTGCTG ACGCGCGCGC GGGCGCGGGG CCTCGTGTGC
bovbeta123  T.cgcTAC.. ...cCTG.T. ACcaagcgcc g.GCcCgggc ag.cgTggtc
```

β_3-AR (primer C Sense)
(576-596)

β_3-AR (primer A Anti-sense)
(596-576)

β_3-AR (primer B Sense)
(576-596)

```
            551                                              600
bovbeta3    CTGGTGTGGG TGGTGTCCGC CGCGGTGTCG TTTGCGCCCA TCATGAGCAA
humbeta3    CTGGTGTGGG TCGTGTCGGC CGCGGTGTCG TTTGCGCCCA TCATGAGCCA
humbeta2    ATGGTGTGGA TTGTGTCAGG CCTTACCTCC TTCTTGCCCA TTCAGATGCA
humbeta1    ACCGTGTGGG CCATCTCGGC CCTGGTGTCC TTCCTGCCCA TCCTCATGCA
bovbeta123  .tgGTGTGGg tcgTgTCgGc C..ggtgTC. TT.g.GCCCA Tc.tgA..cA

            601                                              650
bovbeta3    ATGGTGGCGC ATCGGGGCCG ATGCCGAGGC GCAGCGTTGC CACTCCAACC
humbeta3    GTGGTGGCGC GTAGGGGCCG ACGCCGAGGC GCAGCGCTGC CACTCCAACC
humbeta2    CTGGTACCGG GCCACCCACC A...GGAAGC CATCAACTGC TATGCCAATG
humbeta1    CTGGTGGCGG GCGGAGAGCG A...CGAGGC GCGCCGCTGC TACAACGACC
bovbeta123  cTGGTggCG. g.cggggcCg A...cGAgGC gca.cgcTGC .ActcCaAcc

            651                                              700
bovbeta3    CGCGCTGCTG CACCTTCGCC TCCAACATGC CCTACGCGCT GCTCTCCTCC
humbeta3    CGCGCTGCTG TGCCTTCGCC TCCAACATGC CCTACGTGCT GCTGTCCTCC
humbeta2    AGACCTGCTG TGACTTCTTC ACGAACCAAG CCTATGCCAT TGCCTCTTCC
humbeta1    CCAAGTGCTG CGACTTCGTC ACCAACCGGG CCTACGCCAT CGCCTCGTCC
bovbeta123  cg.gcTGCTG .g.CTTCg.C .CcAAC.tg. CCTAcGc..T g..cTCcTCC
```

β_2-AR (primer E Sense)
(721-741)

β_2-AR (primer D Anti-sense)
(741-721)

```
            701                                              750
bovbeta3    TCGGTCTCGT TCTATCTTCC GCTCCTGGTG ATGCTCTTCG TCTACGCACG
humbeta3    TCCGTCTCCT TCTACCTTCC TCTTCTCGTG ATGCTCTTCG TCTACGCGCG
humbeta2    ATCGTGTCCT TCTACGTTCC CCTGGTGATC ATGGTCTTCG TCTACTCCAG
humbeta1    GTAGTCTCCT TCTACGTGCC CCTGTGCATC ATGGCCTTCG TGTACCTGCG
bovbeta123  t.cGTcTCcT TCTAc.TtC  cCTgct..T. ATG.tCTTcG TcTACgcgcG
```

β_1-AR (primer G Sense)
(724-744)

β_1-AR (primer F Anti-sense)
(744-724)

Figure 19. (cont'd).

```
           751                                                  800
bovbeta3   AGTTTTCGTG GTGGCCACGC GCCAGCTGCG CTTGCTGCGC CGGGAGCTGG
humbeta3   GGTTTTCGTG GTGGCTACGC GCCAGCTGCG CTTGCTGCGC GGGGAGCTGG
humbeta2   GGTCTTTCAG GAGGCCAAAA GGCAGCTCCA GAAGATTGAC AAATCTGAGG
humbeta1   GGTGTTCCGC GAGGCCCAGA AGCAGGTGAA GAAGATCGAC AGCTGCGAGC
bovbeta123 gGTtTTc.tg G.GGCca.g. g.CAGcTgc. ...G.Tg..C agg.ag..Gg

           801                                                  850
bovbeta3   GTCGCTTCCC GCCAGAGGAG TCTCCGCCGG CTCCTTCTCG CTCCGGATCC
humbeta3   GCCGCTTTCC GCCCGAGGAG TCTCCGCCGG CGCCGTCGCG CTCTCTGGCC
humbeta2   GCCGCTTCCA TGTCCAGAAC CTTAGCCAGG TGGAGCAGGA TGGGCGGACG
humbeta1   GCCGTTTCCT CGGCGGCCCA GCGCGGCCGC CCTCGCCCTC GCCCTCGCCC
bovbeta123 GcCGcTTcCc g.ccgaggag tctc.gCcGg cgccg.cgcg ctcccgg.Cc

           851                                                  900
bovbeta3   CCTGGCCTGG CGGGGCCGTG CGCCTCGCCC GCGGGGGTGC CCTCCTACGG
humbeta3   CCGGCCCCGG TGGGGACGTG CGCTCCGCCC GAAGGGGTGC CCGCCTGCGG
humbeta2   GGGCATGGAC TCCGCAGATC TTCCAAGTTC TGCTTGAAGG AGCACAAAGC
humbeta1   GTCCCCGCGC CCGCGCCGCC GCCCGGACCC CCGCGCCCCG CCGCCGCCGC
bovbeta123 .cg.cc.cg. ..ggg.cgt. cgCc.cgccC gcgggggtg. ccgcCtacG.

           901                                                  950
bovbeta3   CCGGCGGCCG GCGCGCCTTC TGCCTCTGCG GGAACACCGC GCCCTGCGCA
humbeta3   CCGGCGGCCC GCGCGCCTCC TGCCTCTCCG GGAACACCGG GCCCTGTGCA
humbeta2   CCTCAAGACG TTAGGCATCA TCATGGGCAC TTTCACCCTC TGCTGGCTGC
humbeta1   CGCCACCGCC CCGCTGGCCA ACGGGCGTGC GGGTAAGCGG CGGCCCTCGC
bovbeta123 Ccg..ggcC. gcgcgcctc. t.cc.c.cc. ggaa.acCg. g.cctg.g..

                                  β3-AR (primer C Anti-sense)
                                  (991-971)
           951                                                  1000
bovbeta3   CCTTGGGGCT CATCATGGGA ACCTTCACTC TCTGCTGGTT GCCTTTCTTT
humbeta3   CCTTGGGTCT CATCATGGGC ACCTTCACTC TCTGCTGGTT GCCCTTCTTT
humbeta2   CCTTCTTCAT CGTTAACATT GTGCATGTGA TCCAGGATAA CCTCATCCGT
humbeta1   GCCTCGTGGC CCTACGCGAG CAGAAGGCGC TCAAGACGCT GGGCATCATC
bovbeta123 cCtT.g.gct CaTcat.gg. ac.t.c.c.c TCt..tggtt gccc.TCttt

           1001                                                 1050
bovbeta3   GTGGTCAACG TGGTGCGCGC CCTCGGGGGC CCCTCTCTGG TGTCCGGCCC
humbeta3   CTGGCCAACG TGCTGCGCGC CCTGGGGGGC CCCTCTCTAG TCCCGGGCCC
humbeta2   AAGGAAGTTT ACATCCTCCT AAATTGGATA GGCTATGTCA ATTCTGGTTT
humbeta1   ATGGGCGTCT TCACGCTCTG CTGGCTGCCC TTCTTCCTGG CCAACGTGGT
bovbeta123 atGG.c..c. t.atgC.Cgc cctgggGggc ccCTctcTgg tctccGgcc.
```

Figure 19. (cont'd).

β_3-AR (primer B Anti-sense)
(1110-1090)

```
             1051                                                  1100
  bovbeta3   CACTTTCCTC GCCCTTAACT GGCTGGGCTA TGCCAACTCT GCCTTCAACC
  humbeta3   GGCTTTCCTT GCCCTGAACT GGCTAGGTTA TGCCAATTCT GCCTTCAACC
  humbeta2   CAATCCCCTT ATCTACTGCC GGAGCCCAGA TTTCAGGATT GCCTTCCAGG
  humbeta1   GAAGGCCTTC CACCGCGAGC TGGTGCCCCA CCGCCTCTTC GTCTTCTTCA
bovbeta123   .a.tt.CcT. gcCctcaac. gGctg..c.A tgcCaact.t GcCTTCaacc
```

β_2-AR (primer E Anti-sense)
(1093-1073)

β_1-AR (primer G Anti-sense)
(1100-1080)

```
             1101                                                  1150
  bovbeta3   CGCTCATCTA CTGCCGCAGC CCGGACTTTC GGAGCGCCTT CCGCCGCCTG
  humbeta3   CGCTCATCTA CTGCCGCAGC CCGGACTTTC GCAGCGCCTT CCGCCGTCTT
  humbeta2   AGCTTCTGTG CCTGCGCAGG TCTTCTTTGA AGGCCTATGG GAATGGCTAC
  humbeta1   ACTGGCTGGG CTACGCCAAC TCGGCCTTCA ACCCCATCAT CTACTGCCGC
bovbeta123   .gctc.T.t. CtgccgCAgc .Cgg.cTTt. ..a.Cgcctt cc.ccGcctc

             1151                                                  1200
  bovbeta3   CTGTGTCGCT GCCGGCCGGA GGAGCACCTC GCCGCTGCCT CCCCGCCCCG
  humbeta3   CTGTGCCGCT GCGGCCGTCG CCTGCCTCCG GAGCCCTGCG CCGCCGCCCG
  humbeta2   TCCAGCAACG GCAACACAGG GGAGCAGAGT GGATATCACG TGGAACAGGA
  humbeta1   AGCCCCGACT TCCGCAAGGC CTTCCAGGGA CTGCTCTGCT GCGCGCGCAG
bovbeta123   ct.tgcc.Ct gCcgc.cggg .g.gCagcg. g.gcc.tgC. ccgcgccccg

             1201                                                  1250
  bovbeta3   AGCCCCCTCC GGCGCCCCCA CGGCCCTGAC CAGCCCCGCT GGCCCCATGC
  humbeta3   CCCGGCCCTC TTCCCCTCGG GCGTTCCTGC GGCCCGGAGC AGCCCAGCGC
  humbeta2   GAAAGAAAAT AAACTGCTGT GTGAAGACCT CCCAGGCACG GAAGACT.TT
  humbeta1   GGCTGCCCGC CGGCGCCACG CGACCCACGG AGACCGGCCG CGCGCCTCGG
bovbeta123   ggc.gccc.c .gcccccc.g .ggcccacgc cgcccg.acg ggc.cctcgc

             1251                                                  1300
  bovbeta3   AGCCCCCAGA GCTCGACGGG GCTTCCTGCG GACTTTCTTA G
  humbeta3   AGCCCAGGGG CTTCTTGGGG AGTTTCTTAG
  humbeta2   GTGGGCCATC AAGGTACTGT GCCTAGCGAT AACATTGATT CACAAGGGAG
  humbeta1   GCTGTCTGGC CCGGCCCGGA CCCCCGCCAT CGCCCGGGGC CGCCTCGGAC
bovbeta123   .gc.ccc.gc cc..tacgGg gc.tc..ga. .aC.ttg.t. c.C...GGA.

             1301                                                  1350
  humbeta2   GAATTGTAGT ACAAATGACT CACTGCTGTA A
  humbeta1   GACGACGACG ACGATGTCGT CGGGGCCACG CCGCCCGCGC GCCTGCTGGA
bovbeta123   GA.....A.. AC.A.....T C...GC.... .CGCCCGCGC GCCTGCTGGA

             1351                                                  1400
  humbeta1   GCCCTGGGCC GGCTGCAACG GCGGGGCGGC GGCGGACAGC GACTCGAGCC
bovbeta123   GCCCTGGGCC GGCTGCAACG GCGGGGCGGC GGCGGACAGC GACTCGAGCC

             1401                                             1447
  humbeta1   TGGACGAGCC GTGCCGCCCC GGCTTCGCCT CGGAATCCAA GGTGTAG
bovbeta123   TGGACGAGCC GTGCCGCCCC GGCTTCGCCT CGGAATCCAA GGTGTAG
```

Figure 19. (cont'd).

Reverse Transcriptase Reaction and cDNA Amplification

RT-PCR assays were performed using the Perkin-Elmer EZrTth RNA PCR Kit, N808-0179, according to the manufacturer's suggested protocols, except as precautionary steps, the experimental RNA samples (porcine satellite and C2C12 RNA) were pretreated with DNase 1 and RNase inhibitor. The Perkin-Elmer Gene® EZrTth RNA PCR Kits were designed to detect gene expression at the mRNA level. The kit provides reagents required to perform the reverse transcription of the mRNA to produce cDNA and the subsequent amplification all in one reaction tube by a single thermostable DNA polymerase rTth DNA polymerase, an enzyme that possesses reverse transcription and DNA polymerization activities under the appropriate buffer conditions. For both reverse transcription and resulting cDNA amplification, master mixes of reagents including buffers, water, dATP, dGTP, dCTP, dTTP, manganese acetate, and rTth DNA polymerase for all samples were prepared and aliquotted (34 μL) to individual 200 μL micro tubes. All assays were done in duplicate. Using such master mixes minimized reagent pipetting errors and variation among assays. The composition of the master mix is present in Table 4. Twelve μL/tube of experimental, pretreated RNA sample from either porcine satellite or C2C12 cells were placed in their respective micro tubes, followed by the addition of 2 μL each sense and antisense primers at a 0.8 μM final concentration (total reaction volume = 50 μL). For the positive control assay, in addition the basal 34 μL master mix, 12 μL H_2O, 1 μL RNA (provided), and 1.5 μL each sense and antisense primers at 0.45 μM final concentration were added to each control assay tube. Briefly, all tubes were centrifuged to remove all bubbles. All tubes were then placed in the Perkin-Elmer

Gene Amp® PCR System 9600 that had been previously checked for functionality. The reverse transcription reactions were conducted at 60° C for 3 min, followed by a two-temperature polymerase chain reaction; cDNA denaturized at 94° C for 15 s and primers annealed and extended at 60° C for 30 s in 40 cycles. The resulting RT-PCR products were delayed at 60° C for 7 min and held at 4° C before removal. Ten μL aliquot samples were taken per cell type per receptor subtype and electrophoresed in a 4% agarose gel in Tris-borate/EDTA running buffer, at 45 amps for 2 h.

Table 4. RT-PCR master mix composition.

Component	μL	Final Composition
Water	11	--
5X EZ Buffer	10	--
dATP (10 mM)	1.5	300 μM
dGTP (10 mM)	1.5	300 μM
dCTP (10 mM)	1.5	300 μM
dTTP (10 mM)	1.5	300 μM
rTth DNA polymerase	2.0	5 units/50 μL
25 mM Mn $(OA_C)_2$ soln.	5.0	2.5 mM

Results and Discussion

The results from the RT-PCR experiments are presented in Figure 20. The positive control RNA and primers (kit provided) produced the expected RT-PCR product of about 300 base pairs (bp) as indicated by its mobility rate compared to the DNA marker V, whose largest band equalled 500 bp. The estimated 300 bp was further confirmed by the DNA sequence chromatogram starting at base 1 to 296 (arrowed). The experimental RNA (porcine and C2C12 cells) produced smaller than expected fragment size (see Table 4). In contrast, no stretch of DNA sequence could

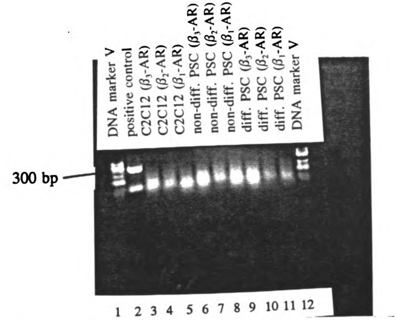


Figure 20. The reverse transcription and DNA amplification products.

Note. The reverse transcription and DNA amplification experiments were performed using seven pairs of primers targeted to recognize all the three mRNA transcripts of βAR. The experiment was conducted as described under the Materials and Methods section. Lane 1) DNA molecular weight marker 5, Lane 2) positive control, Lanes 3-5) C_2C_{12} cells (β_3-AR, β_2-AR and β_1-R), Lanes 6-8) non-differentiated porcine satellite cells (β_3-AR, β_2-AR and β_1-AR), Lanes 9-11) differentiated porcine satellite cells (β_3-AR, β_2-AR and β_1-AR), and Lane 12 DNA molecular weight marker V. These products were resolved in a 4% agarose gel in a Tris-borate/EDTA running buffer at 45 amps for 2 h.

be seen in the differentiated porcine satellite cell RNA-derived RT-PCR chromatogram product. C2C12 cells and non-differentiated porcine satellite cells produced signals that were not discernable from background. The reason(s) for not detecting any βAR transcript, despite all precautionary steps taken, is unclear. Prior to use for the RT-PCR, all RNA samples (porcine satellite and C2C12 cells) were DNase 1-treated and incubated at 37° C for 30 min to degrade all possible contaminating DNA, followed by RNase inhibitor treatment (40 units/μL stock) to deactivate possible contaminating RNases, if any.

Furthermore, experimental RNA was analyzed qualitatively by spectrophotometer and quantitatively using spectrophotometer and gel electrophoresis. The 260-280 absorbance ratio equaled or slightly exceeded 1.8 for all cell types. Ten μg aliquot RNA samples were electrophoresed in a 1.2% agarose gel in 1 X (3-[morpholino]propanesulfonic acid) MOPS, as running buffer, at 45 amps for 2 h (see Figure 21). Because the sequences of the porcine βAR are unknown, and to increase the chances of detecting βAR in C2C12 and porcine satellite cells, multiple primer pairs (sense and antisense) were designed to recognize conserved region in human and bovine β_3-AR. Sequences of β_2-AR and β_1-AR were found to be less conserved between human and bovine. The detection of mRNA and subsequent amplification of

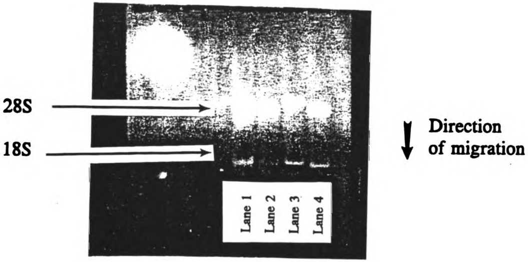


Figure 21. A 1.2% agarose gel showing the 18 and 28S RNA bands.

Note. The figure illustrates porcine satellite and C_2C_{12} cells in four lanes: (a) Lane 1--differentiated C_2C_{12} cells, (b) Lane 2--non-differentiated porcine satellite cells, (c) Lane 3--non-differentiated porcine satellite cells (second preparation), and (d) Lane 4-- differentiated porcine satellite cells. All cell types were grown or differentiated as described in the Materials and Methods section.

the positive control cDNA (see Figure 22) indicated that the thermal cycles and assay conditions worked, which leaves one to speculate that the lack of detection of any βAR transcript in C2C12 and porcine satellite cells (see Figure 23) may be due to the following: (a) Primers may not have been specific enough to detect and discriminate against the βAR subtypes, and (b) βAR were expressed in levels not high enough for detection using this approach.

In the future, the sensitivity or efficiency of this assay may be improved by lowering or raising the anneal/extend temperature of 60° used in this study. The selection of 60° for the anneal/extend temperature was based on the manufacturer's suggested temperature range of 60-72° C. Furthermore, the optimal manganese acetate concentration for RT-PCR using these cells may be determined empirically by varying the manganese acetate concentration. Of course, most importantly, future investigators should consider designing another set of primers highly specific for the porcine βAR if sequences become known.

Conclusion

The radioligand experiments were conducted by incubating increasing concentrations of [^{3}H]CGP12177 with either porcine satellite or C2C12 cell membrane preparations. The specific binding of [^{3}H]CGP12177 to C2C12 membrane preparations was highly specific (90%), saturable, reversible, and of high affinity, as indicated by a low K_D (0.2 nM). Thus the radioligand binding experiment established the presence of βAR in C2C12 cells. For the porcine satellite cell membrane preparation, [^{3}H]CGP12177 binding was linear with increasing protein concentration (20-50 μg protein/mL). This binding, although highly specific (90%) and reversible, was unsaturable. The observed reversibility and specificity of [^{3}H] binding in non-differentiated porcine satellite cells suggested the presence of βAR. These reports were further strengthened by the isoproterenol-induced extracellular cAMP ($P < .001$). In C2C12 cells, 10^{-5} M ISO, RAC, and CLEN significantly increased extracellular cAMP release ($P < .01$). Furthermore, these βAA-induced extracellular cAMP results do not only corroborate the findings from the radioligand binding experiment, indicating the presence of βAR in both cell types, but also establish that these βAR are functional.

We are presenting both biochemical and physiological evidence to indicate the presence of functional βAR in porcine satellite and C2C12 cells. These findings also suggest that C2C12 cells may be used as a muscle cell model to study the mode of action of βAA. Finally, studies that will elucidate the complete sequences of the porcine βAR and the regulatory mechanisms will be helpful in the future.

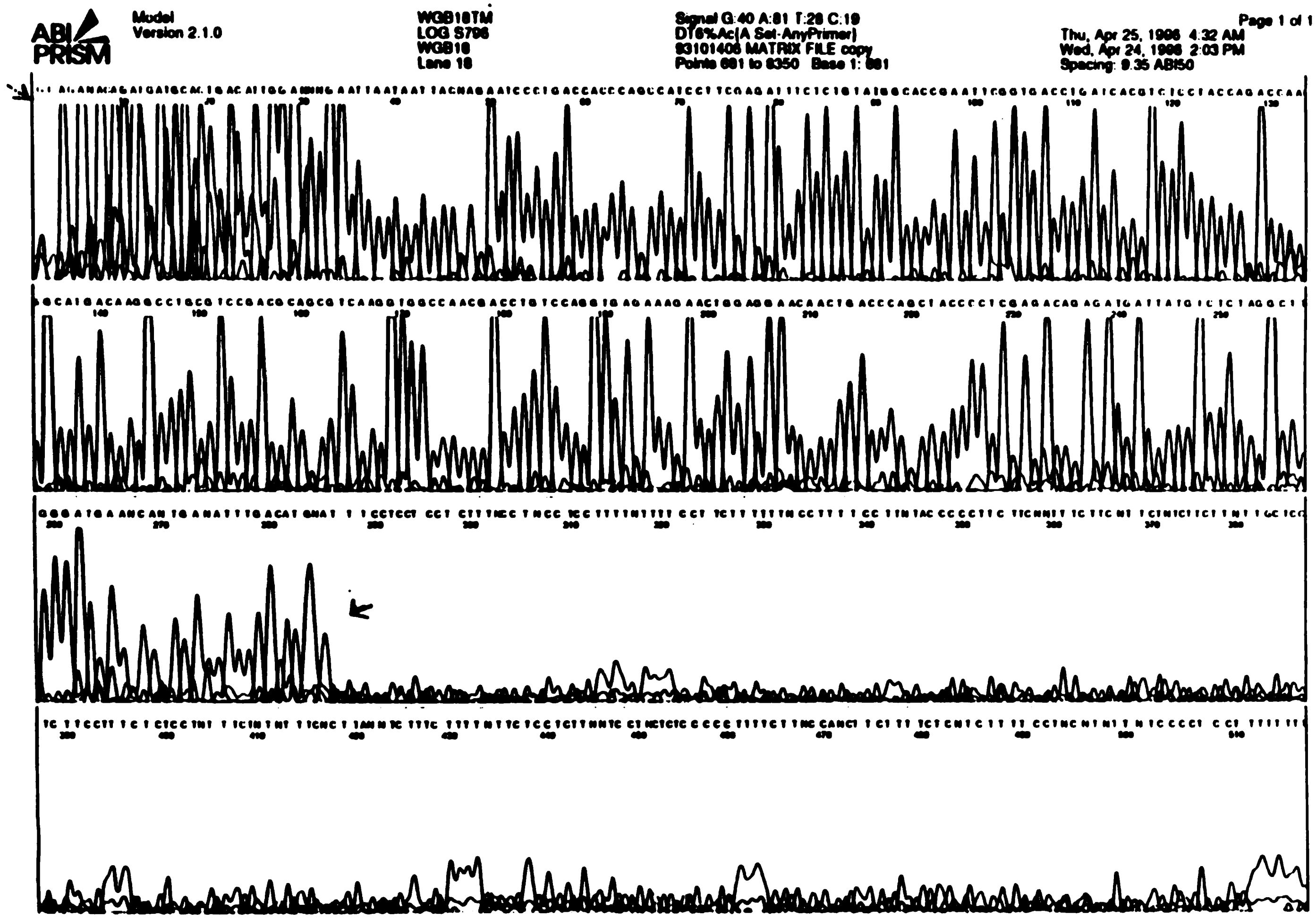


Figure 22. Chromatograph of control RNA-derived RT-PCR product.

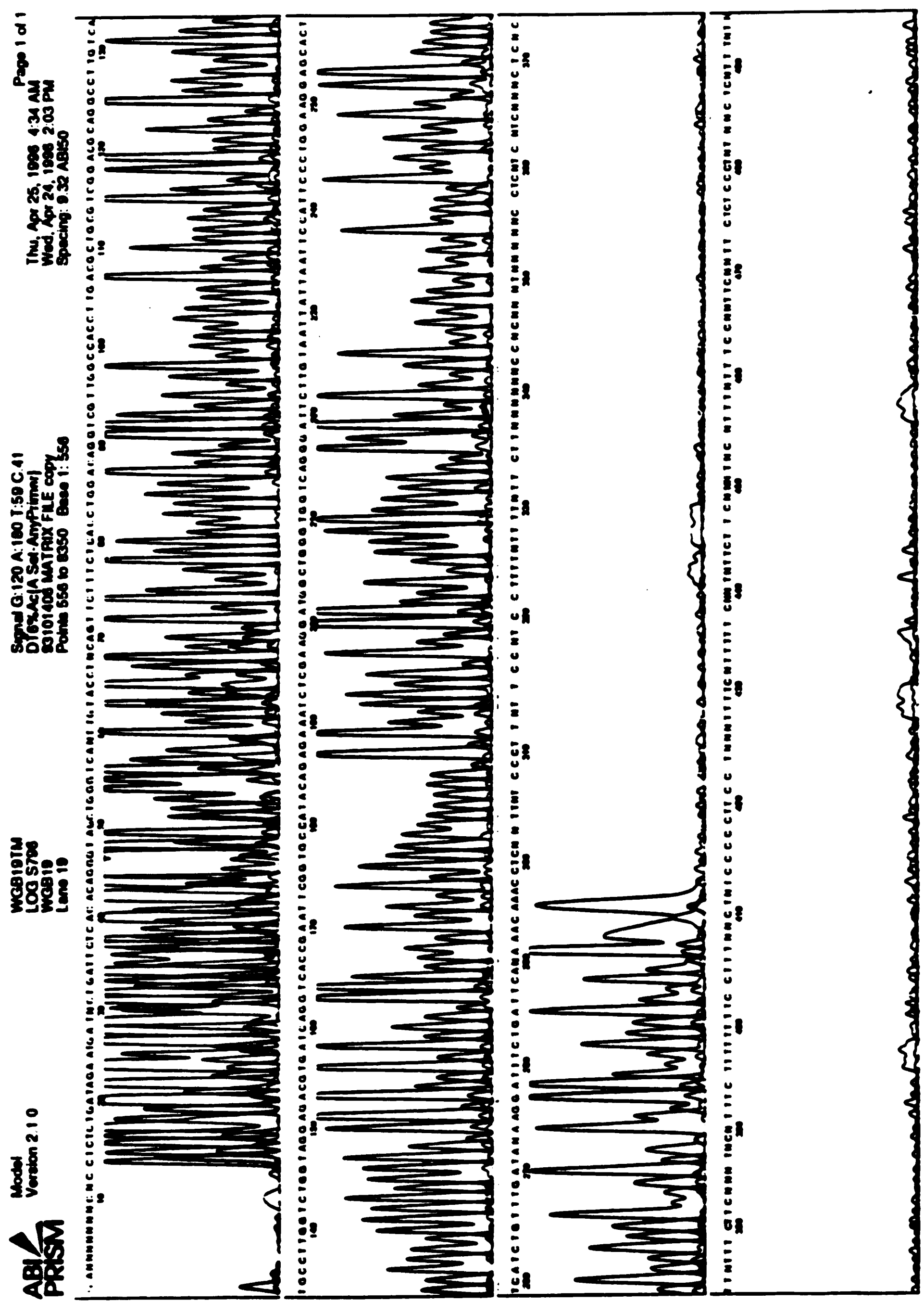


Figure 22. (cont'd).

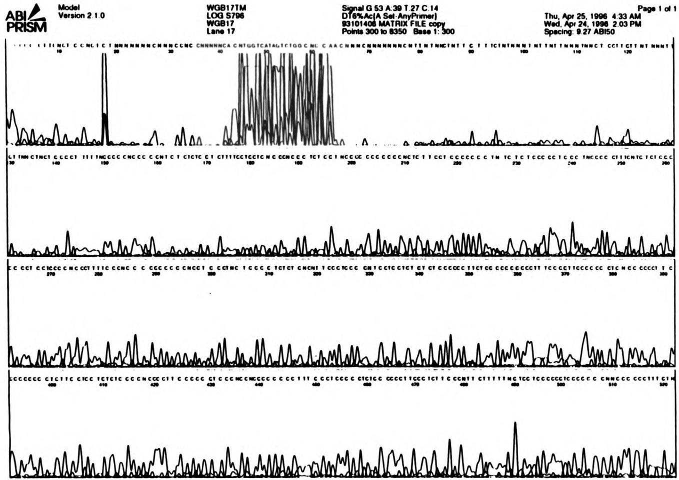


Figure 23. Chromatograph of differentiated porcine satellite cell-derived RT-PCR product.

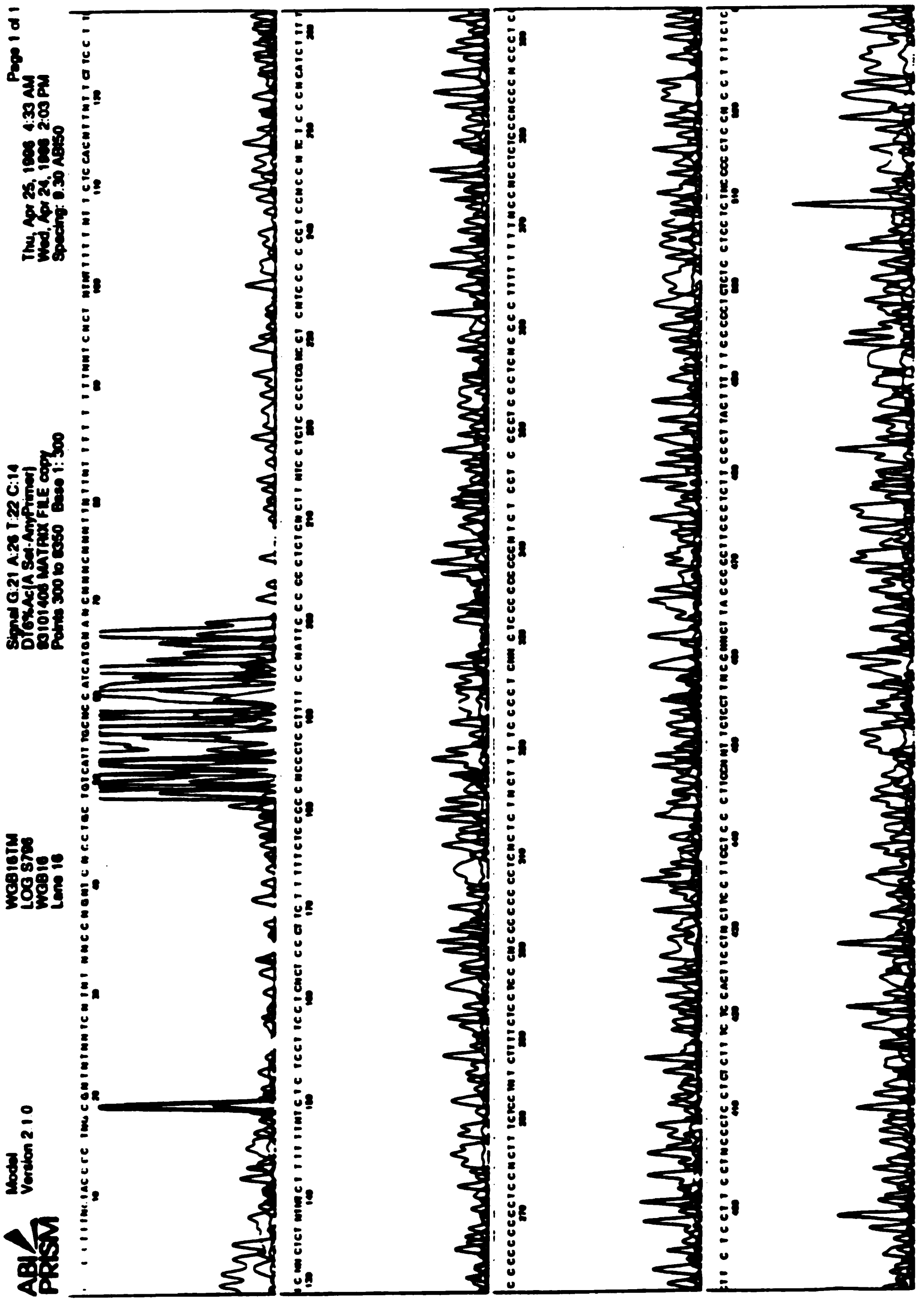


Figure 23. (cont'd).

GLOSSARY

ABAM -- Antibiotic-Antimycotic

ACC -- Acetyl CoA carboxylase

Ara-c -- Cytosine β-D-aribinofuranoside

ATP -- Adenosine triphosphate

β_1AR -- Beta$_1$-adrenergic receptor

β_2AR -- Beta$_2$-adrenergic receptor

β_3AR -- Beta$_3$-adrenergic receptor

βAA -- Beta-adrenergic agonists

βAR -- Beta-adrenergic receptor(s)

βARK -- Beta-adrenergic receptor-associated kinase

β_{max} -- Binding maximum

bFGF -- Basic fibroblast growth factor

cAMP -- Adenosine-3',5'-cyclic monophosphate

cGMP -- Cyclic guanosine monophosphate

CGP12177 -- Ciba Geigy product No. 12177, a hydrophilic β-adrenergic antagonist, 4 [3-[(1,1-Dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2H-benzimidazol-2-one

CIM -- Cimaterol

CLEN -- Clenbuterol

CRE -- cAMP response element

DEPC -- Diethyl pyrocarbonate

DHA -- Dihydroalprenolol

DMEM -- Dulbecco's Modified Eagle Medium

EGF -- Epidermal growth factor

FAR -- Fractional accretion rate

FAS -- Fatty acid synthase

FBR -- Fractional breakdown rate

FBS -- Fetal Bovine Serum

FSR -- Fractional synthesis rate

GDP -- Guanine diphosphate

GH -- Growth hormone

G_i -- The inhibitory alpha subunit of G protein

G_s -- The stimulatory alpha subunit of G-protein

GTP -- Guanine triphosphate

IGF -- Insulin-like growth factor

ISO -- Isoproterenol

K_D -- Dissociation constant

MEM -- Minimum Essential Medium

MOPS -- 3-[N-morpholino] propanesulfonic acid

NAD^+ -- Nicotinamide adenine dinucleotide

NEFA -- Non-esterified fatty acid

NIDDM -- Non-insulin-dependent diabetes melitus

PDGF-AA -- Platelet-derived growth factor-acidic

PDGF-BB -- Platelet-derived growth factor-basic

PKA -- cAMP-dependent protein kinase A

RAC -- Ractopamine

RT-PCR -- Reverse transcriptase-polymerase chain reaction

TBA -- Trenbolone acetate

TBE -- Tris-borate/EDTA

TE -- Tris-EDTA

TGF-β -- Transforming growth factor-Beta

APPENDIX

APPENDIX

Composition of solutions/gels used in characterization,
radioligand binding, cAMP, and RT-PCR studies

1.2% Agarose (RNA Gel) 25 mL

Component	Volume or Weight
Agarose	0.30 g
DEPC-treated milli-Q H_2O	21.75 mL
10X MOPS	2.50 mL
Deionized formaldehyde	0.75 mL

Dissolve agarose in DEPL-treated milli-Q H_2O and 10X MOPS in microwave (approximately 1.5 to 2.0 min, allow to cool to approximately 60° C. Transfer solution to fume hood and slowly add formaldehyde, while mixing, pour gel in hood.

1X RNA Gel Running Buffer (make fresh)

1. 10X MOPS, pH 7.0
2. Dilute 10X MOPS with DEPC-treated milli-Q H_2O (1:10 dilution).

Make solution in a sterile graduated cylinder and store at room temperature until needed.

Denaturing Mix (make fresh)

1.	10X MOPS, pH 7.0	20.0 μL
2.	Deionized formaldehyde	70.0 μL
3.	Deionized formamide	200.0 μL

Make solution in sterile microfuge tube and store at room temperature until needed.

DNA Gel 4% Agarose (40 mL)

Component	Volume or Weight
Agarose	1.6 g
1X TBE	40.0 mL
Ethidium Bromide	1.0 μL

1X DNA Gel Running Buffer (make fresh)

1. 1X TBE as needed.

RNA Sample Preparation

1. Samples should contain at least 3 μg total RNA in a volume of 5.5 μL of TE, pH 8.0 or DEPC-treated H_2O. Sample must be prepared in a sterile microfuge tube.
2. To the RNA samples in 5.5 μL volume add 14.5 μL denaturing mix and cap microfuge tube. Heat denatured samples at 60° C for 5 min.
3. Add 5.0 μL of 4X-dye and apply 25 μL sample to gel.
4. Electrophorese sample at 45 volts for 2-3 h.

MEM for Porcine Satellite Cells (1 L)

1. Dissolve one pack of MEM mix in 500 mL millipore H_2O.
2. Add 2.2 g of sodium bicarbonate.
3. Stir solution for about 5 min or until mixture is completely dissolved.
4. Adjust pH to 7.1 with 1 N HCl.
5. Bring volume to 1 L with millipore H_2O.
6. In a sterile hood, filter medium through 0.2 microns filter paper to sterilize.
7. Keep medium at 4° C until needed.

Growth Medium for Porcine Satellite Cells (500 mL MEM + 10% FBS)

Component	mL
MEM	447.0
FBS	50.0
ABAM	2.5
Gentamicin	0.5

Porcine Satellite Cell Serum-Free Medium

Component	Final Concentration
MEM:MCDB-110 medium	4:1
Dexamethasone	10^{-10} M
Bovine Serum Albumin	0.5 mg/mL
Insulin	10^{-6} M
Transferrin	100 μg/mL
Water-soluble linoleic Acid	0.5μg/mL

DMEM for C2C12 Cells (1 L)

1. Dissolve one pack of DMEM mix in 500 mL millipore H_2O.
2. Add 3.7 g sodium bicarbonate.
3. Stir medium for about 5 min or until mixture is completely dissolved.
4. Adjust pH to 7.3 with 1 N HCl.
5. Bring volume to 1 L with millipore H_2O.
6. In a sterile hood, filter medium through 0.2 microns filter paper to sterilize.
7. Keep medium at 4° C until needed.

Growth Medium for C2C12 Cells (500 mL DMEM + 10% FBS)

Component	mL
DMEM	447.0
FBS	50.0
ABAM	2.5
Gentamicin	0.5

Differentiation Medium for C2C12 Cells (500 mL DMEM + 2% FBS)

Component	Final Concentration or mL
DMEM	487.0
FBS	10.0
ABAM	2.5
Gentamicin	0.5
Insulin	10^{-6} M

500 mL 0.1% Gelatin Solution for Coating Plates/Wells (Porcine Satellite Cell Culture Only)

Component	Volume or Weight
Millipore H_2O	500 mL
Gelatin	0.5 g

1. Autoclave solution for 45 min.
2. In a sterile hood, apply solution to plates/wells, cover tightly, and allow to cool at 4° C for a minimum of 2 h.
3. Return plates/wells to sterile hood, aspirate solution from plates/wells, and rinse with MEM (no serum) equal the volumes of gelatin solution used for coating.
4. Allow MEM (no serum) to remain on plates/wells until cells are ready to be plated.

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