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Gene-Family of Rainbow Trout
(*Oncorhynchus mykiss*)**

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**Molecular Characterization of the β -Adrenoceptor Gene-Family of Rainbow Trout
(*Oncorhynchus mykiss*).**

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This chapter is based on a previously published manuscript that appears in the European Journal of Biochemistry. A Putative β_2 -Adrenoceptor from the Rainbow Trout (*Oncorhynchus mykiss*). Nickerson, J.G., Dugan, S.G., Drouin, G and Moon, T.W. (2001) *Eur. J. Biochem.* **268**, 6465-6472.

Kinetic experiments

In chapters 2 and 3 figure 2.4 (p. 39), figure 3.7 (p. 66) and figure 3.8 (p.67) are based on kinetic experiments performed by S.G. Dugan.

Chapter 4

Tissue and/or RNA samples for hypoxic and cortisol experiments were provided by S.F. Perry. Tissue samples for clenbuterol treated trout were provided by M. Lortie.

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List of Abbreviations

([±]-[R,R]-[4-(2-[3-Chlorophenyl]-2-hydroxyethyl)amino]propyl)phenoxy]acetic acid	BRL
((-)-4-(3-t-butylamino-2-hydroxypropoxy-[5,7] benzimidazol-2-one)	CGP
(±)-1-[2,3-(Dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)]-2-butanol	ICI
Adrenaline	Adr
adrenoceptor	AR
α-adrenoceptor	α-AR
Atenonol	ATL
β-adrenoceptor	β-AR
β-adrenergic receptor kinase	βARK
Bromoacetyl alprenolol menthane	BAAM
Clenbuterol	CLEN
Complete coding sequence	CDS
Dobutamine	DOB
G-protein coupled receptor	GPCR
Gene specific primers	GSP
iodocyanopindolol	CYP
Isoproternol	ISO
Nadalol	NAD
Noradrenaline	Nadr
Polymerase chain reaction	PCR
Proccaterol	PROC
Propanolol	PROP
Protein kinase A	PKA
Protein kniase C	PKC
Rainbow trout	RbT
Random amplification of cDNA ends	RACE
Red blood cell	RBC

Reverse transcription	RT
RNase protection assay	RPA
Sodium proton exchanger	NHE
Transmembrane domain	TMD

Abstract

β -Adrenoceptors (β -ARs) are seven transmembrane domain, G-protein coupled receptors that transduce the cellular effects of the catecholamines, adrenaline (Adr) and noradrenaline (Nadr). Through their interaction with plasma membrane β -ARs, catecholamines modulate a number of key physiological parameters aimed at allowing an organism to cope with environmental and physiological stressors.

Mammalian species express three distinct β -AR subtypes (β_1 -, β_2 - and β_3 -ARs) that exhibit complex modes of regulation and interaction. Relatively few studies have focused on β -ARs from early branching vertebrates such as fish, particularly at the molecular level. The goal of this study was to characterize the β -AR gene-family of the rainbow trout (RbT), *Oncorhynchus mykiss*.

Three putative β -ARs genes were cloned and a phylogenetic analysis predicted one β_2 -AR subtype (RbT β_2 -AR) and two β_3 -AR subtypes (RbT β_{3a} - and RbT β_{3b} -ARs) relative to the established mammalian β -AR classification. The RbT β_2 -, β_{3a} - and β_{3b} -AR genes code for proteins of 409, 427 and 477 amino acids, respectively. Hybridization of gene specific probes to trout tissue RNA indicated that RbT β_2 -AR was highly expressed in the liver, red muscle, and white muscle; RbT β_{3a} -AR was highly expressed in the gill and heart while RbT β_{3b} -AR was highly expressed in the blood. Pharmacological analysis indicated RbT β_2 -AR binding characteristics consistent with mammalian β_2 -ARs while the RbT β_{3b} -AR showed characteristics that were different from all known mammalian β -AR subtypes. Differences in the potential regulatory phosphorylation profiles between trout β -ARs suggests subtype specific sensitivities to the classic mechanisms of β -AR desensitization.

This molecular characterization of trout β -ARs is the first study to demonstrate a β_3 -AR homolog in fish, it identifies the trout red blood cell β -AR as a β_3 -subtype and provides support for the presence of a complex and unique β -AR signaling system in the rainbow trout.

Résumé

Les β -adrénocepteurs (β -ARs) sont des protéines composées de sept domaines transmembranaires et sont couplés aux protéines G permettant ainsi de transmettre le message des catécholamines, soit l'adrénaline (Adr) et la noradrénaline (Nadr), de l'extérieur de la cellule à l'intérieur de la cellule. Par l'entremise des β -ARs de la membrane plasmique, les catécholamines régularisent divers paramètres physiologiques permettant à l'organisme de faire face aux nombreux stress environnementaux et physiologiques.

Les mammifères expriment trois sous-types distincts de β -ARs (β_1 -, β_2 - et β_3 -ARs) qui démontrent des modes complexes de régulations et d'interactions. Peu d'études se sont penchées sur les β -ARs de vertébrés d'embranchement plus ancien tel les poissons, particulièrement au niveau moléculaire. Cette étude a pour but de caractériser la famille de gène des β -ARs de la truite arc-en-ciel (RbT), *Oncorhynchus mykiss*.

Trois gènes potentiels de β -ARs ont été clonés chez la truite arc-en-ciel. Une analyse phylogénique, relative à la classification des mammifères déjà établie, suggère un sous-type β_2 -AR (RbT β_2 -AR) et deux sous-type β_3 -AR (RbT β_{3a} -AR et RbT β_{3b} -AR). Les gènes codant pour les protéines de RbT β -AR sont composés de 409 acides aminés (β_2 -AR), 427 aa (β_{3a} -AR) et 477 aa (β_{3b} -AR). L'hybridation de sondes d'ARN spécifiques pour les gènes de RbT β -ARs indiquent une expression élevée de sous-type β_2 -AR dans le foie, le muscle rouge et le muscle blanc; une expression élevée de sous-type β_{3a} -AR dans les branchies et le cœur, et une expression élevée de sous-type β_{3b} -AR dans le sang. Une analyse pharmacologique de RbT β_2 -AR démontre des caractéristiques ligand-récepteur β_2 -AR conformant à la classification de ces récepteurs chez les mammifères, contrairement aux

caractéristiques de RbT β_{3b} -AR qui diffèrent des mammifères. Des différences au niveau du profil de phosphorylation des β -ARs de truite suggèrent une sensibilité différente selon le sous-type en ce qui a trait aux mécanismes de désensibilisation classique retrouvés chez les mammifères.

Cette caractérisation moléculaire des β -AR de la truite est la première étude à démontrer une protéine β_3 -AR dans les poissons. De plus cette étude identifie le β -AR de globule rouge de truite comme un sous-type β_3 -AR. Finalement, cette étude démontre la présence d'un système de signalisation β -AR complexe et unique chez la truite arc-en-ciel.

Chapter 1: Introduction

General Introduction

The objective of this study was to provide a broad understanding of the characteristics of the rainbow trout β -adrenoceptor (β -AR) gene-family. The primary objectives are to determine the number of β -AR subtypes present in the rainbow trout, the subtype identity of the trout β -ARs relative to the classic mammalian β -AR classification, and the tissue specific expression pattern of trout β -AR subtypes. In order to meet these objectives rainbow trout β -AR cDNAs were cloned and sequenced and phylogenetic methods were employed to determine the subtype identity and evolutionary relationships of the trout β -ARs relative to β -ARs of other vertebrate species. Gene specific probes were then designed to analyze the tissue expression pattern of the various trout β -AR subtypes. A secondary objective was to determine whether trout β -AR mRNA expression patterns are altered by various physiological treatments such as drug administration or environmental hypoxia.

Predictions

Molecular characterization of the rainbow trout β -AR gene-family should indicate a β -AR system of similar complexity to its mammalian counterpart in terms of the number of β -AR subtypes, β -AR subtype tissue expression patterns, and mechanisms of signaling regulation. Furthermore, considerable species differences between trout and mammalian β -AR systems are anticipated at the molecular level. However, characteristics that are fundamental to the functioning of the β -AR system should be conserved through evolution and thus will be present in both trout and mammals.

Rainbow trout as a model organism

The choice of the rainbow trout (*Onchorynchus mykiss*) as an organism of study is based on the extensive use of this species as a model organism in fish physiology. In addition, although the β -ARs of most fish species remain uncharacterized at the physiological level, the β -AR system of the trout has been extensively characterized. Trout tissues where β -AR signaling has been most studied include liver, heart, and red blood cells. In fact, the trout red blood cell may possess the most highly characterized non-mammalian β -AR system at the physiological level. As a result of these previous studies of trout β -ARs, a considerable data set exists that describes the physiology and binding characteristics of trout β -ARs. Our characterization of the β -AR gene-family of the trout at the molecular level will add valuable information to the existing physiological data. In addition, comparing the physiological and molecular data sets should help answer several questions concerning trout β -ARs and the β -AR systems of fish in general.

The β -adrenoceptor system

The purpose of this introduction is to provide general information on the β -ARs obtained from the traditional mammalian perspective. Non-mammalian vertebrates, with specific reference to teleost fish, will then be introduced.

The β -adrenoceptors (β -ARs) represent one of three categories of adrenoceptors that make-up the vertebrate adrenergic signaling system. Adrenoceptors are members of the G-protein coupled receptor (GPCR) super gene-family that is characterized by a seven transmembrane domain secondary structure and coupling to specific second messenger pathways through various G-proteins (Hein and Kobilka, 1995; Strosberg,

1995). The AR signaling system is responsible for transducing the cellular effects of the catecholamines, adrenaline (Adr) and noradrenaline (Nadr) (Hein and Kobilka, 1995; Lomasney *et al.*, 1995; Strosberg, 1995). Adrenaline and Nadr serve dual roles as both hormones and neurotransmitters (Hein and Kobilka, 1995; Strosberg, 1993). In mammals, Adr acts primarily as a hormone and is released from chromaffin cells of the adrenal gland into the blood, where it is transported to peripheral tissues and activates ARs (Lomasney *et al.*, 1995). In contrast, Nadr is primarily released from sympathetic nerve terminals of the autonomic nervous system that innervate various tissues (Lomasney *et al.*, 1995). The tissue specific physiological responses elicited by AR activation assist an organism in coping with environmental and physiological stressors (Reid *et al.*, 1998). Our work focuses on β -ARs in their role as hormone receptors and does not describe the parasympathetic nervous system.

The Mammalian β -Adrenoceptor Model

Adrenoceptor classification

Adrenoceptors were first classified into two categories, α or β based on the rank order potency of six sympathomimetic amines for adrenergic responses in different tissues (Ahlquist, 1948). As new biochemical and molecular techniques became available this original classification was expanded to presently include three broad AR categories, α_1 , α_2 , and β with each category further broken down into subtypes giving a final classification of α_{1a} -, α_{1b} -, and α_{1c} -AR; α_{2a} -, α_{2b} -, and α_{2c} -AR; and β_1 -, β_2 -, and β_3 -AR (Rohrer, 1998). Molecular biological data confirms the presence of nine distinct AR genes in several mammalian genomes (Liggett and Raymond, 1993; Milligan *et al.*, 1994;

Lomasney *et al.*, 1995). Each category of AR couples to a different class of G-protein and therefore activates different signal transduction pathways that bring about different cellular responses to Adr/Nadr (Milligan *et al.*, 1994; Lomasney *et al.*, 1995). Activation of α_1 -AR subtypes results in increased intracellular Ca^{2+} levels through a G_q -protein; α_2 -AR subtypes act via a G_i -protein to inhibit adenylyl cyclase activity and reduce intracellular cAMP levels; and, β -AR subtypes couple to a G_s -protein to stimulate adenylyl cyclase activity and increase intracellular cAMP levels (Lomasney *et al.*, 1995). Biochemical and molecular biological data indicate close functional and evolutionary relationships between adrenoceptor subtypes within a particular category. However, a broad evolutionary analysis of several GPCR family members including adrenergic, octopaminergic, dopaminergic and muscarinic receptors does not support the presence of a monophyletic AR gene-family (Fryxell, 1995). Phylogenetic analysis of an alignment containing the sequences of twenty ARs, seventeen muscarinic receptors, eleven dopamine receptors, and one octopamine receptor supports the presence of a monophyletic catecholamine receptor gene-family composed of receptors for dopamine and Adr/Nadr (Fryxell, 1995). Within this catecholamine receptor gene-family, α_2 -ARs group with dopamine type D2, D3, and D4 receptors, β -ARs group with D1 and D5 dopamine receptor subtypes, and α_1 -ARs are in a separate group by themselves. These results suggest that the ability to bind Adr/Nadr evolved three separate times during the course of adrenoceptor evolution (Fryxell, 1995).

Tertiary structure

β -Adrenoceptors show structural and functional hallmarks characteristic of the GPCR super gene-family. The mammalian β_2 -AR has been extensively characterized at the molecular and biochemical levels and serves as a general structural and functional model for all other neuro/hormone receptors in the GPCR super gene-family (Hein and Kobilka, 1995). Structurally the β -ARs are composed of seven membrane spanning domains (TMDs) of 21-28 hydrophobic amino acids, three extracellular and three intracellular loops of varying lengths, an amino terminal extracellular tail, and a carboxy terminal intracellular tail (Figure 1.1) (Strosberg, 1993; Hein and Kobilka, 1995; Summers *et al.*, 1997). Comparison of β -AR subtypes indicates that the TMDs are highly conserved in length and sequence (70-80%) while the intracellular/extracellular loops and the amino and carboxy tails have divergent sequences and lengths (Lomasney *et al.*, 1995; Strosberg, 1995). The divergence in sequence and length between β -AR subtypes at the level of the third intracellular loop and cytoplasmic tail are particularly significant as these receptor regions play a major role in β -AR/G-protein interaction and regulation of β -AR signaling activity (Liggett and Raymond, 1993; Valdenaire and Vernier, 1997; Ferguson, 2001).

Studies employing various molecular techniques such as photoaffinity labeling, site directed mutagenesis, and chimeric receptor molecules have led to the elucidation of the β -AR ligand-binding pocket, site of G-protein coupling, and the position of regulatory phosphorylation sites (Liggett and Raymond, 1993; Strosberg, 1993; Valdenaire and Vernier, 1997; Ferguson, 2001). The ligand binding pocket of the β -ARs is located

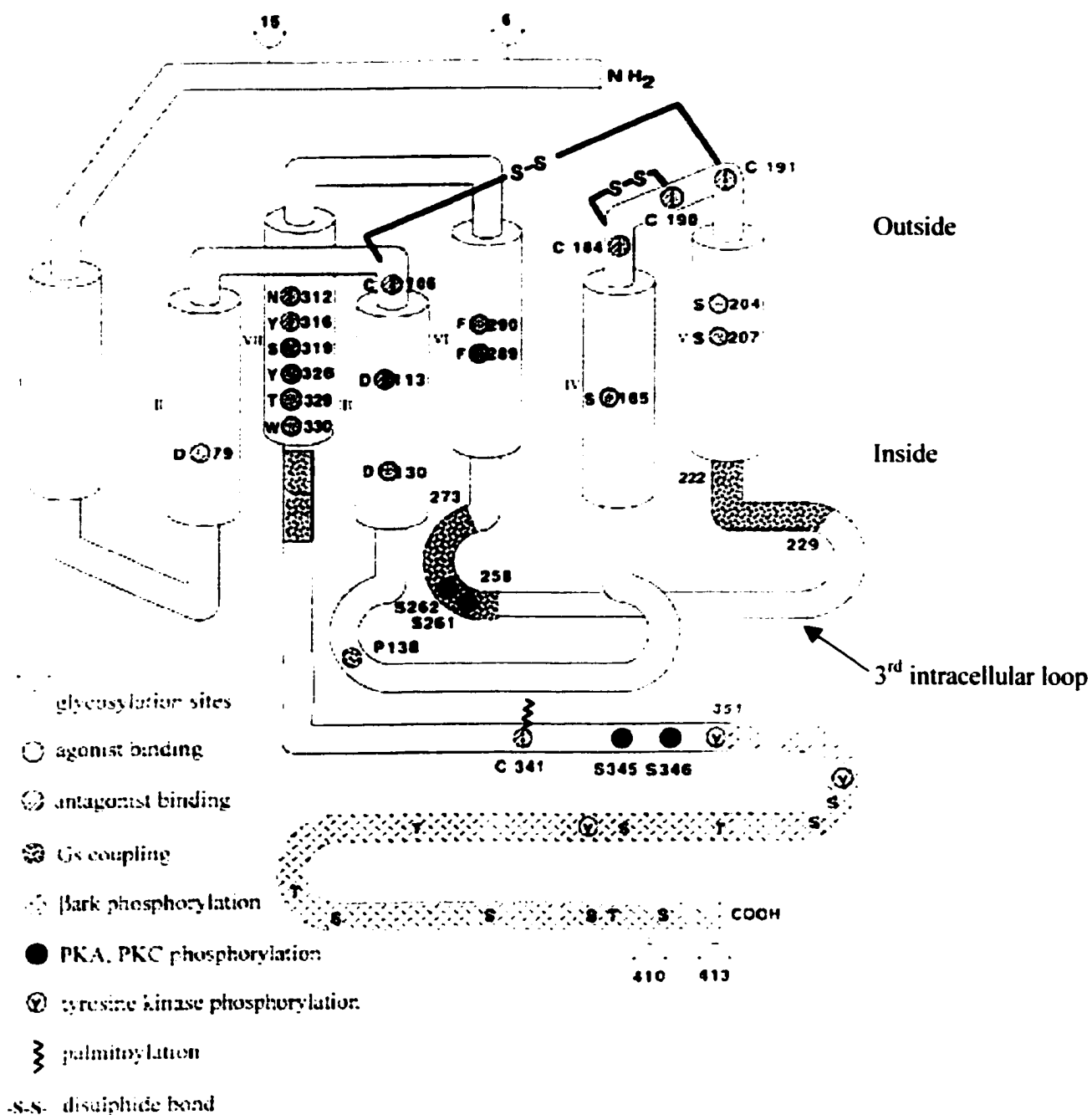


Figure 1.1 Diagrammatic representation of the current model of the β_2 -adrenoceptor showing major functional domains. Larger diameter sections represent membrane-spanning domains (I-VII), the NH₂ tail is outside of the cell and the COOH tail is inside the cell. Amino acids are identified using the single letter convention and key amino acids are numbered. Figure modified from Summers *et al.* (1997).

within the hydrophobic core of the receptor and is formed by the arrangement of the seven TMDs (Figure 1.1) (Liggett and Raymond, 1993; Strosberg, 1993; Valdenaire and Vernier, 1997); the amino and carboxy terminal tails play no apparent role in ligand binding as removing most of these regions has no effect on ligand binding to β -ARs (Ostrowski *et al.*, 1992; Strosberg and Marullo, 1992). Protection and mutational studies indicate that a single G-protein contacts the β -AR structure at three sites, two of these are located within the third intracellular loop and the third is located in the cytoplasmic tail in close proximity to the seventh TMD (Figure 1.1) (Strosberg, 1997). Several putative protein kinase A (PKA)/protein kinase C (PKC) and β -adrenergic receptor kinase (β ARK) phosphorylation sites are located in close proximity to these areas of β -AR/G-protein interaction (Figure 1.1). The apparent association of putative phosphorylation sites with receptor regions which contact the G-protein is not surprising given the role of phosphorylation in the regulation of β -AR signaling activity (see below).

Signal transduction

Adrenaline and Nadr are hydrophobic molecules and therefore are unable to freely cross cell membranes; yet, these hormones are capable of effecting rapid intracellular changes. The intracellular affects of Adr and Nadr are mediated by ARs through a process known as signal transduction that is common to all members of the GPCR super gene-family (Valdenaire and Vernier, 1997; Ferguson, 2001). Characterization of the signal transduction pathways of several GPCRs has led to a general model of signal transduction composed of a membrane bound receptor, a trimeric guanine nucleotide binding protein (G-protein), an effector enzyme and a second messenger. The signaling pathway of the mammalian β_2 -AR was one of the first

transduction pathways to be characterized and serves as a model of GPCR signaling (Hein and Kobilka, 1995). Extensive characterization of mammalian β -ARs indicates that the same "typical" β -AR signaling pathway for all three subtypes involves a stimulatory class of G-protein (G_s -protein), adenylyl cyclase (effector), cAMP (second messenger), and cAMP-dependent protein kinase A (PKA) (Figure 1.2). β -AR signaling begins with activation of plasma membrane β -ARs by the binding of Adr/Nadr at the ligand-binding pocket. Plasma membrane β -ARs spontaneously isomerize between an active and an inactive conformation; in the absence of ligand an equilibrium exists between these two conformations that favors the lower energy inactive conformation and negligible background signaling occurs (Valdenaire and Vernier, 1997). β -Adrenoceptor agonists such as the endogenous hormones Adr and Nadr recognize, bind to, and stabilize β -ARs in their active conformation causing a shift in the equilibrium towards the active conformation (Valdenaire and Vernier, 1997). This is in contrast to antagonists that recognize and stabilize β -ARs in their inactive conformation thus preventing β -AR signaling. Once the receptor is activated, a trimeric G-protein composed of α , β , and γ subunits, assembles at the receptor G-protein binding domain located on the intracellular surface of the receptor (Figure 1.1) (Valdenaire and Vernier, 1997). β -Adrenoceptors interact with a G-protein that has a stimulatory effect on the enzyme adenylyl cyclase: G-proteins that have this stimulatory effect are classified as stimulatory G-proteins (G_s -proteins) (Milligan *et al.*, 1994; Lomasney *et al.*, 1995). When the G_s -protein associates with the β -AR, the guanine-nucleotide binding site located on the G_s -protein α subunit is occupied by GDP; coupling between G_s and AR catalyzes the exchange of this GDP molecule for GTP thus activating the G_s -protein (Lomasney *et al.*, 1995; Valdenaire and

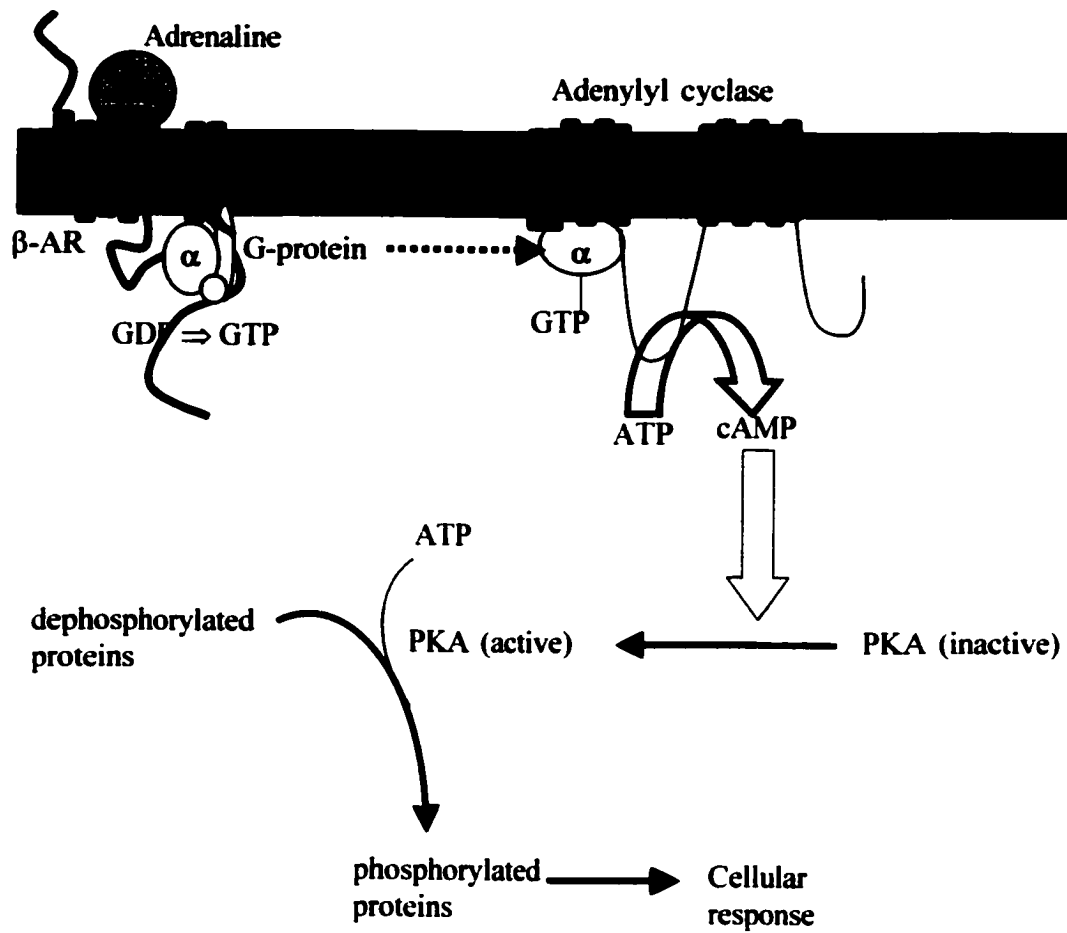


Figure 1.2 Classical signal transduction pathway of mammalian β -ARs.

Vernier, 1997; Kompa *et al.*, 1999; McDonald and Lefkowitz, 2001). The activated G_s-protein disassociates from the β-AR and splits into G_sα-GTP and G_sβ/γ portions. The G_sα-GTP portion translocates along the inner surface of the cell membrane until it encounters and binds to the membrane bound form of adenylyl cyclase. Binding of G_sα-GTP activates the enzyme adenylyl cyclase that catalyzes the production of cAMP from ATP (Milligan *et al.*, 1994; Lomasney *et al.*, 1995; Kompa *et al.*, 1999). Increasing levels of intracellular cAMP results in the activation of PKA, an enzyme that is able to modulate the activity of numerous cellular proteins by catalyzing specific serine-tyrosine phosphorylation (Exton, 1979; Bristow *et al.*, 1990; Mersmann, 1998). Thus, it is ultimately through PKA-mediated phosphorylation of cellular proteins that Adr/Nadr are able to exert an effect on a number of cellular processes in a wide variety of tissues. It should be noted that alternate β-AR signaling pathways do exist (Hein and Kobilka, 1995; Xiao and Lakatta, 1995; Gauthier *et al.*, 1996). For example, the opening of cardiac myocyte Ca²⁺ channels requires β₁-AR activation, but can occur independently of phosphorylation; this is thought to occur by G_s-β/γ interaction with the channel protein (Xiao *et al.*, 1994; Kuschel *et al.*, 1999). In addition all three β-AR subtypes have the ability to couple to non-G_s G-proteins *in vitro* suggesting that β-ARs may be capable of activating different signal transduction pathways *in vivo* (Hein and Kobilka, 1995; Xiao and Lakatta, 1995; Gauthier *et al.*, 1996; Rohrer, 1998). The involvement of several intermediates in β-AR signaling provides an opportunity for both signal amplification and regulation of signaling. Activation of a single β-AR leads to activation of multiple G_s-proteins, these in turn activate several adenylyl cyclase molecules causing rapid increases in intracellular cAMP levels bringing about the activation of multiple PKA

molecules: this process is known as signal amplification and is how Adr at 10^{-7} M in the blood can cause glucose concentrations in the cell/blood to rise in excess of 10^{-3} M. At the level of regulation it appears that the β -AR is the major target of the mechanisms controlling signaling (Lomasney *et al.*, 1995; Valdenaire and Vernier, 1997).

Regulation of signaling

Due to the physiological importance and high energy costs of many of the cellular responses associated with β -AR activation, signaling by these receptors is stringently controlled. Characterization of mammalian β -AR signaling indicates that the receptor is the major target of mechanisms that regulate signaling and these regulatory mechanisms account for both the rapid termination of signaling after agonist removal and the blunting of responses caused by chronic agonist exposure (Lomasney *et al.*, 1995; Marullo *et al.*, 1995; Valdenaire and Vernier, 1997). Regulation of β -AR signaling is achieved by three processes: uncoupling of the β -AR/G-protein interaction; sequestration of β -ARs onto internal cellular membranes; and, down-regulation of β -AR protein (Liggett and Raymond, 1993; Lomasney *et al.*, 1995). These three processes are shown in Figure 1.3. All three processes are phosphorylation-dependent and each operates on a different time scale. Uncoupling of the receptor from the G-protein is a result of PKA or PKC mediated phosphorylation of residues in the third intracellular loop and cytoplasmic tail regions of the receptor and this can occur within seconds of β -AR activation. Phosphorylation of the β -AR in the vicinity of the G-protein binding domain prevents G-protein entry into this site and signaling is terminated (Figure 1.3) (Liggett and Raymond, 1993; Strosberg, 1993; Lomasney *et al.*, 1995; Valdenaire and Vernier, 1997; Tsao and von Zastrow, 2000; Ferguson, 2001). Regulation of β -AR signaling by sequestration involves the

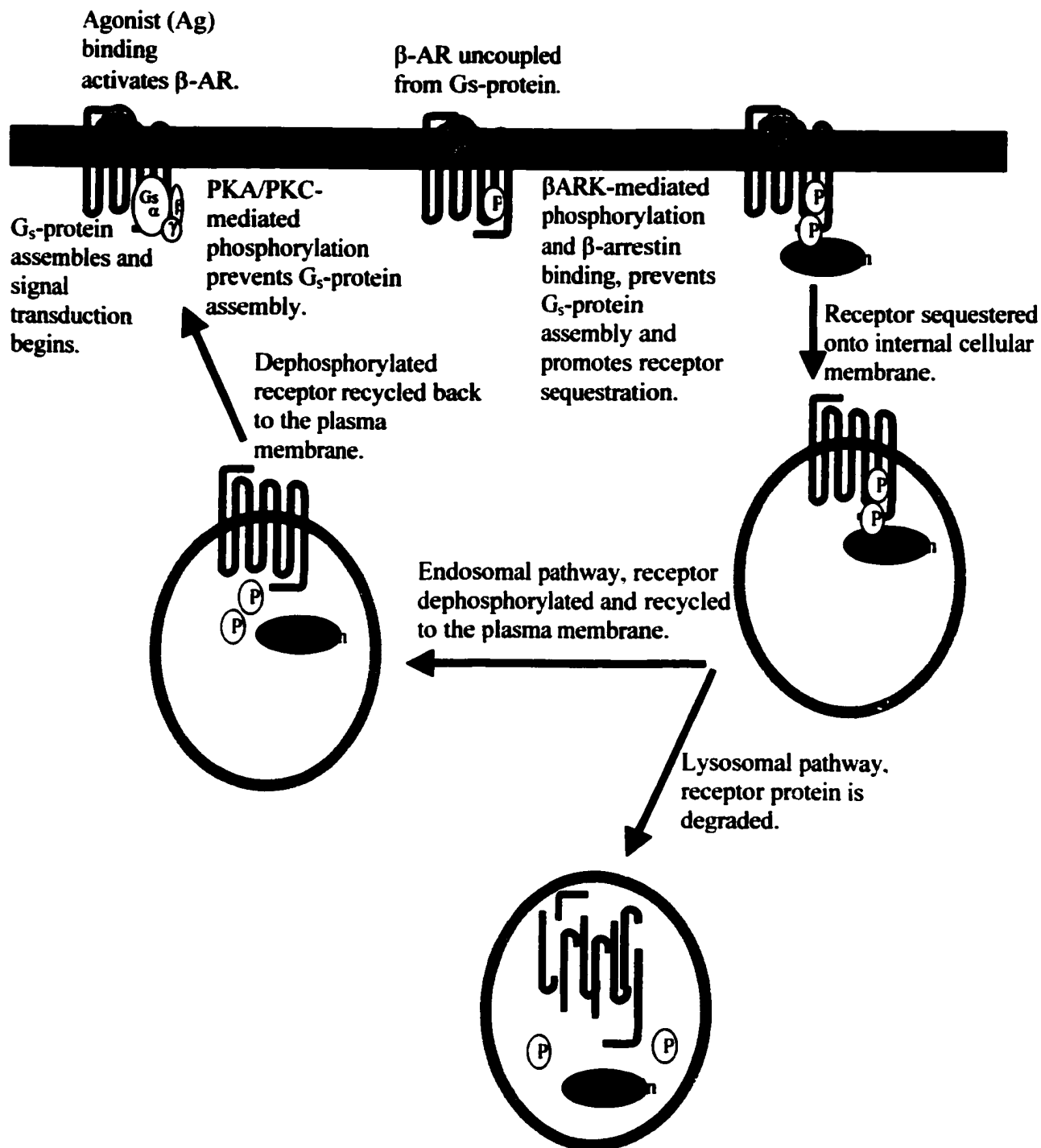


Figure 1.3 Schematic representation of the mammalian model of β -AR signal regulation mediated by PKA/PKC and β ARK phosphorylation of the receptor. Figure modified from McDonald and Lefkowitz (2001).

internalization of β -ARs from the plasma membrane onto membranes of internal cellular vesicles with the internalized receptors being unavailable for activation by agonist binding. The process of sequestration occurs within minutes to hours of β -AR activation and can occur by either PKA/PKC or β ARK mediated pathways. PKA/PKC and β ARK recognize and phosphorylate different sets of β -AR cytoplasmic tail phosphorylation sites to trigger sequestration (Figure 1.1) (Strosberg, 1993; Hein and Kobilka, 1995; Valdenaire and Vernier, 1997; Tsao and von Zastrow, 2000). Phosphorylation by PKA/PKC is sufficient to cause receptor sequestration, whereas, β ARK mediated uncoupling and sequestration requires receptor phosphorylation and subsequent binding of the accessory protein, β -arrestin to trigger sequestration (Strosberg, 1993; Lomasney *et al.*, 1995; Valdenaire and Vernier, 1997; McDonald and Lefkowitz, 2001). These phosphorylation events lead to the migration of β -ARs to clathrin coated pits where they are internalized and at this point the internalized receptors may be recycled to the plasma membrane or be subject to proteolytic cleavage (Valdenaire and Vernier, 1997; Ferguson, 2001; McDonald and Lefkowitz, 2001). If plasma membrane β -ARs do not remain active then the sequestered receptors are shunted into the endosomal pathway where they are dephosphorylated and recycled back to the plasma membrane. However, if the agonist remains and plasma membrane β -ARs continue to be active, then the sequestered receptors enter into the final regulatory pathway known as down-regulation (Lomasney *et al.*, 1995; Valdenaire and Vernier, 1997; Tsao and von Zastrow, 2000; Ferguson, 2001; McDonald and Lefkowitz, 2001). Down-regulation occurs after prolonged β -AR activation of hours to days and results in the loss of β -AR protein (Lomasney *et al.*, 1995; Tsao and von Zastrow, 2000). Down-regulation occurs by increased rates of proteolytic

decay and/or reduction of β -AR mRNA (Lomasney *et al.*, 1995; Tsao and von Zastrow, 2000; Ferguson, 2001). Down-regulation by proteolytic decay is achieved by trafficking of sequestered β -ARs into the lysosomal pathway where the receptor proteins are destroyed by proteolytic cleavage (Tsao and von Zastrow, 2000; Ferguson, 2001). Reduction of β -AR mRNA can occur by either reducing the rate of β -AR gene transcription or by destabilizing β -AR mRNA (Liggett and Raymond, 1993; Hein and Kobilka, 1995; Lomasney *et al.*, 1995; Marullo *et al.*, 1995). Together these mechanisms of uncoupling, sequestration, and down-regulation combine to produce reduced tissue sensitivity to Adr/Nadr, a process called desensitization (Liggett and Raymond, 1993; Freedman *et al.*, 1995; Hein and Kobilka, 1995).

As a result of the ubiquitous nature of PKA/PKC activation and the substrate specificity of β ARK, β -AR desensitization can occur in a homologous or heterologous manner. Homologous desensitization of β -ARs is mediated by β ARK phosphorylation, while heterologous desensitization is mediated by PKA/PKC phosphorylation (Hein and Kobilka, 1995; Lomansey *et al.*, 1995; Valdenaire and Vernier, 1997; Mayor *et al.*, 1998). β ARK is activated specifically as a result of β -AR signaling and β ARK phosphorylates only active β -ARs, thus β ARK-mediated desensitization is referred to as homologous because it is dependent upon β -AR activation (Hein and Kobilka, 1995; Valdenaire and Vernier, 1997). In contrast PKA and PKC activity is stimulated by a wide variety of signaling systems and these kinases phosphorylate both active and inactive β -ARs; thus, PKA/PKC-mediated desensitization can occur independent of β -AR activation and is referred to as heterologous desensitization (Hein and Kobilka, 1995; Valdenaire and Vernier, 1997).

β -Adrenoceptors of Early Branching Vertebrates.

During periods of environmental or physiological stress the catecholamines, Adr and Nadr, are released from chromaffin cells and sympathetic nerve terminals and elicit a number of tissue specific responses collectively aimed at allowing the organism to cope with the stressor (Perry and Reid, 1993; Gamperl *et al.*, 1994; Fabbri and Moon, 1994; Reid *et al.*, 1998; Vatner *et al.*, 2000). Through their interaction with the different AR subtypes present in various tissues, Adr and Nadr are capable of affecting major changes in several key physiological parameters (Randall and Perry, 1992; Perry and Reid, 1993; Strosberg, 1993). In mammals control of the adrenergic response in a particular tissue tends to be dominated by a single adrenoceptor subtype. The adrenoceptor subtype that dominates the response of a particular tissue can be species specific. For example, catecholamine-induced glucose release from the liver is predominantly under the control of β_2 -ARs in guinea pigs, whereas, in the rat it is predominantly controlled by α_1 -AR (Sulakhe *et al.*, 1988). Despite these species differences the adrenergic response of several mammalian tissues have become associated with signaling through β -ARs. Examples of adrenergic responses that tend to be dominated by β -AR signaling in mammals include β_2 -AR-mediated glucose release from hepatocytes, β_1 -AR-mediated increases in heart rate and force of contraction, β_1 -AR-mediated dilation of blood vessels, and β_1 - and β_3 -AR-mediated thermogenic responses in brown and white adipose tissue. The physiological importance of the β -AR system as a mechanism for coping with environmental and/or physiological disturbances is demonstrated by the conservation of this system throughout the course of vertebrate evolution. The presence of β -ARs has been previously demonstrated by molecular biological or pharmacological means in

species throughout the vertebrate phylum from the earliest branching lampreys and hagfish (Vincent *et al.*, 1998) to teleost fishes (Janssens and Lowery, 1987; Fabbri *et al.*, 1992; 1995; Dugan and Moon, 1998), amphibians (Sulakhe *et al.*, 1988; Janssens and Grigg, 1992; Devic *et al.*, 1997), reptiles (Sulakhe *et al.*, 1988; Janssens and Grigg, 1992), birds (Yarden *et al.*, 1986; Chen *et al.*, 1994), and mammals (Wolfe *et al.*, 1976; Kawai *et al.*, 1986; Sulakhe *et al.*, 1988; García-Sáinz *et al.*, 1996). Molecular cloning of partial β -AR sequences from tissues of the sea lamprey and hagfish supports the presence of β -ARs in these earliest branching vertebrate lineages and additional molecular studies have produced full-length β -AR clones from the turkey (Yarden *et al.*, 1986; Chen *et al.*, 1994) and toad (Devic *et al.*, 1997). These studies reveal the conservation of several β -AR molecular characteristics during vertebrate evolution including a seven transmembrane domain secondary structure, a conserved location of receptor/G-protein interaction, and the presence of potential sites for PKA/PKC and β ARK-mediated phosphorylation. With the exception of these few molecular studies, most evidence supporting the presence of β -ARs in the tissues of non-mammalian vertebrates has come from physiological/pharmacological studies. Studies of early branching vertebrates including fish indicate that many of the tissue responses associated with β -AR signaling in mammals are also mediated through β -ARs in these non-mammalian vertebrates including increased rate and force of cardiac contraction (Ask *et al.*, 1980; 1981; Keen *et al.*, 1993; Gamperl *et al.*, 1994), glucose release from the liver (Wright *et al.*, 1987; Fabbri *et al.*, 1992; 2001; Reid *et al.*, 1992; Hemmings and Storey, 1994), and dilation of blood vessels (Nilsson, 1983; Axelsson *et al.*, 1994; Sundin, 1995). In addition, novel tissue β -AR responses have evolved in some non-mammalian vertebrates. An example

of one such response is the rainbow trout red blood cell where signaling through a β -AR modulates hemoglobin/oxygen binding affinity by altering red cell intracellular pH (Borgese *et al.*, 1992; Nikinmaa, 1992; Guizouarn *et al.*, 1993; Reid *et al.*, 1993; Perry *et al.*, 1996). Characterization of non-mammalian β -ARs at the molecular and physiological levels not only demonstrates the presence of β -ARs in non-mammalian vertebrates but also highlights the presence of subtle differences between mammalian and non-mammalian β -AR systems. Some of these differences likely represent adaptations of the β -AR system in a particular vertebrate group to cope with a specific set of environmental/physiological challenges while other differences are likely due to neutral evolutionary changes between vertebrate groups.

Within the vertebrate phylum, and particularly in mammals, catecholamines have a well known positive chronotropic and inotropic effect on the heart mediated by cardiac β -ARs (Bristow *et al.*, 1990; Rohrer, 1998; Bristow, 2000). The catecholamine-induced increase in cardiac output is designed to maintain or increase oxygen delivery to the tissues during stress (Randall and Perry, 1992; Reid *et al.*, 1998). The presence of cardiac β -ARs has been previously shown in several non-mammalian vertebrates such as birds, frog and a number of teleost species (Ask *et al.*, 1980; Rocha-Singh *et al.*, 1991; Gamperl *et al.*, 1994; Skeberdis *et al.*, 1997; Olsson *et al.*, 2000) and as in mammals, these cardiac β -ARs are responsible for transducing the positive effects of catecholamines on cardiac performance (Ask, 1983; Farrell *et al.*, 1986). However, in contrast to the mammalian heart where a β_1 -AR subtype generally dominates, β -AR signaling in the hearts of many teleosts, including the rainbow trout, is dominated by a β_2 -AR (Ask, 1983; Gamperl *et al.*, 1994). Furthermore, although the β_1 -AR tends to be

the dominant receptor, β_2 - and β_3 -ARs are also reported in mammalian hearts whereas the hearts of most teleosts studied to date contain exclusively the β_2 -AR subtype based on pharmacology (Gamperl *et al.*, 1994). It should be noted that a recent study of teleost cardiac myocyte β -ARs indicates an approximate three-fold species difference in K_d values between eight teleost species. The authors propose that these differences in K_d values among the different species might be due to the presence of different β -AR subtypes on myocytes from different species (Olsson *et al.*, 2000). It is interesting to note that teleost cardiac β -ARs may differ in their sensitivity towards down-regulation relative to mammalian and avian cardiac β -ARs (Gamperl *et al.*, 1998). Prolonged elevation of plasma catecholamine levels caused by acute stress produce a well documented down-regulation of cardiac β -ARs in mammals and birds. This occurs as a result of reduced cardiac β -AR density so that the positive effects of catecholamines on cardiac performance are greatly reduced and catecholamines may be unable to maintain or enhance cardiac performance during subsequent bouts of stress (Kompa *et al.*, 1999; Bristow, 2000; Ostrom *et al.*, 2000). In contrast to the mammalian and avian heart, exposure of trout cardiac myocytes to short-term acute hypoxia did not produce the expected reduction in cell surface β -ARs (Gamperl *et al.*, 1998). The resistance to down-regulation of the trout cardiac β -AR is also suggested by an earlier study where repeated bolus injections of catecholamines produced no detectable reduction in cell surface β -ARs (Gamperl *et al.*, 1994). Taken together these studies provide support for the presence of a down-regulation-resistant β -AR in the heart of the trout and the authors speculate that this resistance towards down-regulation may represent an adaptation of the cardiac β -AR system in teleosts to routine exposure to hypoxia (Gamperl *et al.*, 1998).

The potential adaptation of the trout cardiac β -AR system to resist down-regulation is tissue specific as a number of treatments including hypoxia, injection of catecholamines, and exhaustive exercise produce obvious down-regulation of β -ARs in other trout tissues such as the liver and the red blood cell (Perry *et al.*, 1996; Dugan, S.G. and Moon, T.W. unpublished). In addition to hypoxia, fish often encounter conditions of changing temperatures that have also been shown to have an effect on teleost cardiac β -ARs. For example, ventricles from trout acclimated to 8°C possessed more β -ARs than ventricles from trout acclimated to 18°C (Keen *et al.*, 1993). The presence of seasonal differences in the dominant cardiac AR subtype has been demonstrated in the American eel where an α -AR dominates during winter acclimation, but β -AR dominates during summer acclimation (Peyraud-Waitzenegger *et al.*, 1980).

In addition to their stimulatory effects on cardiac performance, Adr/Nadr elicit significant metabolic responses primarily at the level of energy metabolism (Randall and Perry, 1992; Strosberg, 1993; Fabbri and Moon, 1994). The most important metabolic response in terms of coping with an acute stressor is the mobilization of glucose from the liver into the blood for transport to peripheral tissues where it is used to fuel increased metabolism (Randall and Perry, 1992; Fabbri and Moon, 1994; Reid *et al.*, 1998). In mammals, the well-established hyperglycemic effects of catecholamines are initiated by these hormones stimulating glycogenolysis and gluconeogenesis in the liver, while at the same time inhibiting hepatic glycolysis through activation of glycogen phosphorylase and inhibition of pyruvate kinase (Wright *et al.*, 1987; Fabbri *et al.*, 1995). In fish as well as other early branching vertebrate lineages, Adr/Nadr have the same hyperglycemic effect

as seen in mammals and there is no evidence to suggest mechanistic differences between vertebrate groups for these effects (Fabbri *et al.*, 1999).

While the effect of Adr/Nadr on glucose metabolism is highly conserved throughout vertebrate evolution the same cannot be said for the AR subtype that mediates this response. As noted above there is considerable mammalian species variation in the AR subtype that controls glucose release from the liver, including α_1 - β -ARs or a combination of α_1 - and β -ARs (Fabbri and Moon, 1995; Nonogaki and Iguchi, 1997; Nonogaki, 2000). Previous studies indicate that control of catecholamine-elicited glucose release from avian livers shows the same species specific variability between α_1 - and/or β -ARs as in mammals (Sulakhe *et al.*, 1988). In amphibians and reptiles the β -AR pathway appears to dominate control of hepatic glucose release while hepatic α_1 -ARs are either absent or present only at levels near the limit of detection (Janssens and Grigg, 1988; Sulakhe *et al.*, 1988; Hemmings and Storey, 1994). Studies to characterize hepatic ARs in fish indicate the presence of a functional β -AR pathway linked to glucose release in all species studied including the rainbow trout, goldfish, carp, catfish, brown bullhead, rockfish, American eel, toadfish, and lungfish with no evidence to support a role for hepatic α_1 -AR in several of these same species (Danulat and Mommsen, 1990; Fabbri *et al.*, 1995; 1998; 2001; Krumschnabel *et al.*, 2001). The presence of hepatic α_1 -ARs has been shown for some fish species including goldfish, catfish, bullhead, American eel, and rainbow trout (Brighenti *et al.*, 1987; Fabbri *et al.*, 1992; 1995; Zhang *et al.*, 1992; Fabbri and Moon, 1994; Krumschnabel *et al.*, 2001). However, the role of the α_1 -AR pathway in mediating glucose release from the liver of fishes has only been shown definitively in the goldfish, catfish, and American eel (Fabbri *et al.*, 1993; 1995; Krumschnabel *et al.*,

2001). Interestingly, although rainbow trout hepatocytes appear to possess more α_1 -ARs than β -ARs there is no evidence to support the presence of a functional α_1 -AR signal transduction pathway in trout hepatocytes so the physiological significance of hepatic α_1 -ARs in this species remains equivocal (Fabbri and Moon, 1995). Previous pharmacological studies to characterize hepatic β -ARs reveal that in most teleosts studied to date a β_2 -AR subtype mediates signaling in the liver. However, the pharmacological characteristics of hepatic β -ARs from rock-fish indicate the presence of a hepatic β_1 -AR subtype (Danulat and Mommsen, 1990).

In summary, the hepatic β -AR system shows a broad distribution throughout the vertebrate phylum and plays a role in mediating glucose release from the liver of species from all major vertebrate groups (Janssens and Grigg, 1988; Sulakhe *et al.*, 1988; Nonogaki, 2000). A hepatic α_1 -AR system mediating glucose release shows a broad distribution only in mammalian and avian groups, a functional α_1 -AR pathway has not been demonstrated in reptiles or amphibian livers and although α_1 -ARs have been demonstrated on hepatocytes from some fish species, α_1 -AR mediated glucose release has only been demonstrated in three fish species. The broad distribution of the hepatic β -AR pathway in all vertebrate lineages and the sporadic distribution of a functional hepatic α_1 -AR pathway in early branching vertebrate lineages led to the hypothesis that the hepatic β -AR system was more primitive than the α_1 -AR system (Fabbri and Moon, 1994). This hypothesis received further support from developmental studies of the rat where fetal and neonate livers are dominated by β -ARs while adult rat livers are dominated by α_1 -ARs (McMillan *et al.*, 1983). This hypothesis has been called into

question with the recent finding of α_1 -AR-mediated glucose release from the livers of three fish species (Fabbri *et al.*, 1993; 1995; Krumschnabel *et al.*, 2001); however, the importance of the hepatic α_1 -AR pathway in most fish species remains in question and further work is needed in order to refute or support the proposed primitive origin of the hepatic β -AR.

Many of the catecholamine elicited tissue specific responses mediated by β -ARs including enhanced cardiac performance and hyperglycemia occur in organisms throughout the vertebrate phylum (Janssens and Grigg, 1988; Sulakhe *et al.*, 1988; Fabbri and Moon, 1994; Fabbri *et al.*, 1998; Olsson *et al.*, 2000). In addition to these highly conserved and broadly distributed β -AR-mediated responses, novel β -AR responses also exist in some vertebrate groups as a result of their exposure to a specific set of environmental/physiological stressors. Perhaps the most highly characterized of these non-mammalian novel β -AR responses is the β -AR/sodium proton exchanger (β -AR/ β NHE) system present in the red blood cell (RBC) of some teleost fishes (Randall and Perry, 1992). The rainbow trout RBC β -AR/ β NHE system has been extensively studied at the physiological level and serves as a model for this system. In the trout catecholamines are able to alter hemoglobin/oxygen binding affinity by controlling the activity of the RBC β NHE through β -AR signaling (Nikinmaa, 1992; Randall and Perry, 1992; Perry and McDonald, 1993; Perry *et al.*, 1996). Signaling through RBC β -AR leads to accumulation of cAMP and activation of PKA that in turn activates β NHE by phosphorylation (Borgese *et al.*, 1992; Randall and Perry, 1992; Perry and McDonald, 1993). Once activated the β NHE extrudes H^+ from the RBC in exchange for Na^+ resulting in the alkalization of the RBC cytoplasm which causes an increase in

hemoglobin affinity for oxygen and allows for increased transport of oxygen by the blood (Randall and Perry, 1992; Perry and McDonald, 1993). Previous physiological studies of trout RBC β -AR indicate that this receptor shows binding characteristics of a traditional mammalian β_1 -AR subtype (Perry and McDonald, 1993). Studies of the RBC of several teleost species indicate that the β -AR/ β NHE system is found in several groups of fish (Thomas and Perry, 1992)

Due to the involvement of β -ARs in human medical conditions such as heart disease and obesity, the β -AR system of mammals has been exhaustively studied at the molecular, biochemical and pharmacological levels as evidenced by over 130 entries of β -AR sequences in Genbank and the development of numerous β -AR subtype selective agonists and antagonists acting as pharmaceuticals. Together these studies provide a comprehensive picture of the highly complex and flexible β -AR signaling system present in mammalian tissues. However, our understanding of the β -AR systems from earlier vertebrate lineages remains limited particularly at the molecular and pharmacological levels. With the exception of the turkey β_1 - and β_{4c} -ARs (Yarden *et al.*, 1986; Chen *et al.*, 1994) and the toad β_1 -AR (Devic *et al.*, 1997), no molecular characterization of β -ARs are reported from early branching vertebrates. Most studies of fish β -ARs have focused on the physiology and pharmacology of these receptors. Typically these studies employ synthetic agonist/antagonists known to be selective for a particular mammalian β -AR subtype in order to determine the subtype identity of β -ARs controlling the adrenergic response in a particular fish tissue. While these studies have provided evidence for a β -AR system in fishes, they are limited by the fact that the pharmacological agents used are specific for mammalian β -AR subtypes and may not

effectively differentiate between different fish β -AR subtypes. Thus many questions remain unanswered regarding the number and identity of β -AR subtypes, tissue specific patterns of expression, modes of signal regulation, and pharmacological binding properties of fish β -ARs. In order to provide answers to some of these questions the β -AR gene-family of the rainbow trout will be characterized at the molecular level in this thesis. My work indicates that the trout β -AR gene-family is composed of at least three members, one homologue of mammalian β_2 -AR and two homologs to the mammalian β_3 -AR. The trout β_2 - and β_{3a} -ARs exhibit a broad tissue expression pattern while the trout β_{3b} -AR is expressed specifically in the blood. The phosphorylation profiles of trout β -ARs suggests that regulation of β -AR signaling occurs by the mechanisms of uncoupling, sequestration, and down-regulation. However, the different trout β -AR subtypes show varying degrees of sensitivities towards these mechanisms. Taken together the results of this study support the presence of a β -AR system in the rainbow trout with similar complexity to that of its mammalian counterpart.

Chapter 2: Characterization of the Rainbow trout β_2 -Adrenoceptor Gene

Introduction

The β -adrenoceptors are members of a family of seven transmembrane domain receptors coupled to G-proteins that transduce the cellular effects of the stress hormones adrenaline and noradrenaline (Strosberg, 1993; Mayor *et al.*, 1998). The typical β -AR signal transduction pathway results in increased cellular cAMP levels caused by G_s -protein mediated stimulation of adenylyl cyclase activity (Mersmann, 1998; Rohrer, 1998). The second messenger, cAMP, regulates the activity of several key enzymes, most notably protein kinase A, that modulates the activity of a number of proteins within the cell to bring about a tissue specific response (Strosberg, 1992; Gamperl *et al.*, 1994). The various tissue responses elicited by catecholamine release are aimed at allowing organisms to cope with environmental and physiological stressors (Gamperl *et al.*, 1994; Reid *et al.*, 1998; Vatner *et al.*, 2000).

Due to the implication of the β -ARs in several human disorders such as heart disease and obesity (Davies *et al.*, 1996; Li *et al.*, 1996; Emilsson *et al.*, 1998; Vatner *et al.*, 2000), the role of β -ARs in the stress response has been well characterized in several mammalian tissues. Increased cardiac output, elevated blood glucose, increased thermogenesis in brown adipose tissue and vasodilation of systemic blood vessels are some of the common adrenergic responses typically associated with activation of β -ARs in mammals (Freedman *et al.*, 1995; Rohrer, 1998). Pharmacological and molecular biological studies in mammals indicate the existence of three closely related genes coding for β_1 -, β_2 -, and β_3 -AR subtypes (Strosberg, 1993; Granneman, 2001).

Characterization of the β -ARs in tissues of early branching vertebrates, such as fish, has occurred predominantly at the pharmacological level. With the exception of *Xenopus* and turkey (Yarden *et al.*, 1986; Chen *et al.*, 1994; Devic *et al.*, 1997), no studies have dealt with the molecular biology of non-mammalian β -ARs. Physiology-based studies using several species of fishes reveal that some of the typical mammalian β -adrenergic responses including increased cardiac output and elevated blood glucose levels also occur in fish through agonist mediated β -AR binding (Reid *et al.*, 1998; Fabbri *et al.*, 2001). In addition, fish β -ARs elicit responses unique to particular species or groups of fish. For example, catecholamine release causes an increase in hemoglobin/oxygen binding affinity in the rainbow trout erythrocyte by activation of a red blood cell β -AR (Perry and Reid, 1993).

In spite of the many studies dealing with the physiology and pharmacology of fish β -ARs, many fundamental questions remain concerning the number of β -AR genes in fishes, the pattern of expression of these genes, and their regulation. To address some of these questions we have cloned a putative β_2 -AR from the rainbow trout (*Oncorhynchus mykiss*) and studied its expression pattern in eight tissues. This information is the first step towards a broader understanding of fish β -AR systems and provides further insight into the phylogenetic relationships between vertebrate β -ARs.

Materials and Methods

Animals

Rainbow trout, *Oncorhynchus mykiss* Walbaum, were obtained from Linwood Acres Trout Farm (Campellcroft, Ontario) and were transported to the University of Ottawa. Fish were acclimated at least six weeks in fiberglass tanks containing 1275 L of aerated dechloraminated City of Ottawa tap water at 12°C with a 12L:12D photoperiod. Fish were fed commercial trout pellets (Purina trout chow) five times a week.

Isolation of RNA

Total cellular RNA was isolated from fresh tissues of the rainbow trout using Trizol reagent (GibcoBRL, Burlington, ON, Canada) according to the protocol provided by the manufacturer. RNA concentrations and quality were verified using spectrophotometry and gel electrophoresis. Aliquots of RNA to be used in RT-PCR or RNase protection assays were treated with DNase 1, DNA Free™ (Ambion Austin, Tx, USA) before use.

Amplification of RbT β_2 -AR cDNA

An initial set of trout β_2 -AR clones, spanning the first to sixth transmembrane domains (approximately 750 bp) were amplified using a nested RT-PCR strategy. Oligo-p(dT)₁₅ primed cDNA was synthesized using the 1st Strand cDNA Synthesis Kit for RT-PCR (Roche Molecular Biologicals, Laval, QC, Canada). A preliminary round of PCR amplification was performed using degenerate primers AdrUni 5' and AdrUni 3' (Table 2.1) followed by a second round of amplification using nested degenerate primers BetaUni 5' and BetaUni 3' (Table 2.1). The sequence of these clones was then used to design gene specific primers (GSPs) for 5' and 3' RACE.

Table 2.1 Primers used to amplify various fragments of the RbT β_2 -AR.

	5' primers ^a	Pos ^b	3' primers	Pos ^c	temp ^e °C
AdUni	YTITGYVYIHHTIDSIXTIGAYMGITA	9-18	RTADATIAXIGGRTTIADISHISWRTT	334-324	45
BetaUni	GGIAAYBTIYTIQTIXAT	53-59	CCARCAIARISWRAAIRYICCCATDAT	294-286	45
RACE GSP1			CACACACACAGACATCCGCCGCCGT	120-113	
RACE GSP2			GCCAGGTGTTGAGGAGGATGTAGC	102-96	47
RACE GSP3			GGTGATGAACATGTTGGTGACC	84-76	47
RACE GSP4	CACCAACCGCTGCCCTACGCCGTAGC	197-208			55
RACE GSP5	CAGGAGGCCAGGAAGCAGCTGG	227-233			60
RPA probe	ATTAGGTGACACTATAGAAGVGG	257-262	TAATACGACTCACTATAGGAGACAGAG	293-287	50
	GGGACCGGAGCGCGGAGG		GSARAAGRYGCCCAT		
GSP 6	ATGGAGAATGTGAGCAGCCTGCC	1-8			55
GSP 7			ATATTCTTATAGTAATGC	3' UTR ^d	55

^a Primer DNA sequences are given according to the IUPAC code

^b Position of primer relative to the RbT β_2 -AR amino acid sequence presented in figure 2.1.

^c Annealing temperature used in PCR amplification.

^d GSP 7 anneals to a region within the 3' untranslated region of the gene.

RACE PCR

The 5' and 3' RACE System for Rapid Amplification of cDNA Ends, Version 2 (GibcoBRL) was used to amplify the 5' and 3' ends of the trout β_2 -AR cDNA. In the 5' RACE protocol, trout β_2 -AR gene specific primer 1, GSP1 (Table 2.1), was used to prime cDNA synthesis. This cDNA was then used as the template in an initial round of PCR amplification using a second trout β_2 -AR gene specific primer, GSP2 (Table 2.1) and the 5' amplification primer provided with the kit. A 5 μ L aliquot of this initial PCR amplification was then used as a template for a second round of nested PCR using a third trout β_2 -AR gene specific primer 3, GSP3 (Table 2.1) and the abridged universal amplification primer (AUAP) provided with the kit.

Synthesis of cDNA for 3' RACE was primed with the 3' amplification primer provided in the kit. A first round of PCR amplification was performed using trout β_2 -AR gene specific primer 4, GSP4 (Table 2.1) and the AUAP. A second round of semi-nested PCR amplification using trout β_2 -AR gene specific primer 5, GSP5 (Table 2.1) and the AUAP was then carried out.

The complete coding region of the trout β_2 -AR was PCR amplified using non-degenerate primers, GSP 6 and GSP 7 (Table 2.1), designed from the sequence of the β_2 -AR 5' and 3' RACE clones. To ensure sequence accuracy the full length RbT β_2 -AR gene was cloned six times from different PCR amplifications and each clone was sequenced on both strands.

All PCR amplifications described above used the following regimen of denaturing, annealing and extension: 1 x 2 min at 94°C, 30 x (30 sec at 94°C, 30 sec at

45°C-60°C, 1 min at 72°C), and 1 x 10 min at 72°C. Annealing temperatures varied from 45°C to 60°C depending on the primers being used (Table 2.1).

Prediction of phosphorylation profile

NetPhos 2.0 (Blom *et al.*, 1999) (<http://www.cbs.dtu.dk/services/NetPhos/>) was used to predict phosphorylation of serine, threonine, and tyrosine residues in the third intracellular loop and intracellular tail regions of the RbT β_2 -AR.

Phylogenetic analysis

The RbT β_2 -AR amino acid sequence was aligned with Genbank sequences of various β -AR subtypes from selected organisms using the default settings in CLUSTAL W version 1.8 (Thompson *et al.*, 1994). The accession numbers for sequences used in the phylogenetic analysis are as follows; amphioxus d/ β -AR (CAA06536), bovine β_1 -AR (AAF21435), bovine β_2 -AR (Q28044), bovine β_3 -AR (P46626), mouse β_1 -AR (449413), mouse β_2 -AR (P18762), mouse β_3 -AR (P25962), frog β_1 -AR (O42574), human β_1 -AR (NP000675), human β_2 -AR (AAB82149), human β_3 -AR (AAA35550), rat β_1 -AR (S12591), rat β_2 -AR (NP036624), rat β_3 -AR (P26255), turkey β_1 -AR (P07700) and turkey β_{4c} -AR (P43141). The extracellular and intracellular tails of the β -ARs were excluded from the phylogenetic analysis due to their lack of sequence conservation. The portion of the sequences used for phylogenetic analysis spanned from the conserved isoleucine at the amino end of the first transmembrane domain to the conserved arginine at the carboxy end of the seventh transmembrane domain (see Figure 2.1). Maximum likelihood phylogenetic analysis was performed using PUZZLE version 4.0.2 (Strimmer and von Haeseler, 1996). The following program settings were used; quartet puzzling tree search, compute exact quartet likelihood, 1000 puzzling steps, use the amphioxus

dopamine/ β -AR sequence as outgroup, branch lengths are not clocklike. JTT model of substitution, amino acid frequencies were estimated from the data set and the model of rate heterogeneity was 1 invariable + 8 gamma rates.

RNase Protection Assay

The expression pattern of the RbT β_2 -AR gene was determined using the RNase protection assay, RPAIII™ (Ambion). Eight tissues were used; gill, heart, kidney, liver, red muscle, white muscle, blood, and spleen. The template used to synthesize the probe for the RNase protection assay experiments was obtained by PCR amplifying the 3rd intercellular loop region from a plasmid clone of the RbT β_2 -AR (bases 778 to 880). The primers used to amplify probe template incorporated the promoter sequences for T7 and SP6 RNA polymerases (Table 2.1) so that antisense or sense RNA probes could be transcribed. Radiolabeled antisense RNA probe was transcribed using MAXIscript™ (Ambion) with T7 RNA polymerase and ³²P-UTP (Amersham Pharmacia, Baie d'Urfé, QC, Canada). Full-length probe was purified from a denaturing 4% polyacrylamide, 8 M urea gel. Approximately 4.2×10^4 cpm of probe were hybridized to 25 μ g of total RNA for approximately 16 h at 42°C. Nonhybridized transcripts were digested with approximately 0.4 units of RNase A and 15 units of RNase T1 (cloned) at 37°C for 90 mins. Protected fragments were resolved on a denaturing 6% polyacrylamide, 8 M urea gel that was dried and subjected to autoradiography. This RPA analysis was repeated on three separate individuals

Membrane preparation

Fish were killed by a blow to the head and the liver quickly excised. Semi-purified membranes were prepared using a modified method developed for chick hearts (Daveloose *et al.*, 1993). The liver was rinsed in Hanks' buffered saline (in mM: 136.7

NaCl, 5.4 KCl, 0.8 MgSO₄, 0.33 NaH₂PO₄, 0.44 KH₂PO₄, 5.0 Hepes, 5.0 Hepes-Na, and 1.0 NaHCO₃, pH 7.63), resuspended in 8 volumes of homogenization buffer (in mM: 0.8 MgCl₂, 10 Hepes, 0.1 PMSF, pH 7.6) and homogenized with a Polytron homogenizer (Brinkmann Instruments). The homogenate was centrifuged for 10 min at 10°C and 2000 g (Sorvall RG-5B plus, SS 34 rotor). The resulting supernatant was again centrifuged for 20 min at 38,000 g, the supernatant was removed and the pellet resuspended in incubation buffer (in mM: 50 Tris, 10 MgCl₂, pH 7.6) and again centrifuged for 15 min at 38,000 g three times. The final pellet was resuspended in a small volume of incubation buffer, centrifuged for 1 min at 14,000 g (Eppendorf 5414c) and the pellet frozen at -80°C for future studies.

Binding assays

Frozen trout membrane pellets were resuspended in a small volume of incubation buffer and protein content was determined using the Bicinchoninic Acid (BCA) assay (Pierce) with bovine serum albumen as standard. Trout β -adrenoceptors were characterized using the hydrophilic antagonist ((-)-4-(3-t-butylamino-2-hydroxypropoxy-5,7-³H]benzimidazol-2-one) ³H-CGP-12177 (³H-CGP; Amersham, specific activity 50.0 Ci mmol⁻¹). Fifty μ L of membranes (containing approximately 230 μ g protein) were incubated for 1 h at room temperature (19 \pm 1°C) in a final volume of 150 μ L in the presence of a saturating concentration of ³H-CGP (approximately 1 nM) alone or with increasing concentrations of selective and non-selective agonists and antagonists. In order to prevent photodegradation, incubations were done in the dark and aluminum foil was used to cover the microcentrifuge tubes containing the ligands. Assays were terminated by four repeated washings of ice cold 0.9% NaCl with a cell membrane harvester (Brandel 24R). Filters (#32 glass fibre filters, Schleicher and Schuell) were

placed in scintillation vials containing 4 mL scintillation cocktail (Safety-Solve; RPI, Mount Prospect, IL, USA). Radioactivity was determined using a Packard 2500 TR liquid scintillation counter after the vials were allowed to sit in the dark for at least 24 h. K_d and K_i values were calculated using the EBDA and LIGAND computer programs (Munson and Rodbard, 1980).

Results

The RbT β_2 -AR gene codes for a 409 amino acid protein that has the same seven transmembrane domain structure as its mammalian counterparts. Alignment of the RbT β_2 -AR amino acid sequence with β -AR subtypes from various vertebrate species shows a high degree of sequence conservation between all subtypes and species particularly within the transmembrane domains (data not shown). The RbT β_2 -AR shows the highest level of average amino acid identity, 63%, with mammalian the β_2 -AR, followed by 57% with the mammalian β_1 -AR and 51% with the mammalian β_3 -AR. In a multiple sequence alignment of the RbT and mammalian β_2 -AR amino acid sequences, most of the sequence conservation occurs within the transmembrane domains while the highest levels of divergence occur in the amino and carboxyl tail regions (Figure 2.1). The RbT β_2 -AR protein sequence lacks potential phosphorylation sites within its G-protein binding domains, these sites are conserved among the mammalian β_2 -AR sequences (Figure 2.1). Three potential phosphorylation sites were identified in the carboxyl terminus of the RbT β_2 -AR, but only 1 of these sites (position 366) aligned with a similar site in the mammalian β_2 -ARs. Phylogenetic analysis of the β -AR sequence alignment using a maximum likelihood method produced a tree consisting of three major clades: a β_1 -AR clade, a β_2 -AR clade, and a β_3 -AR clade; the RbT β_2 -AR sequence is placed at the base of the β_2 -AR clade (Figure 2.2). The tree topology has high support values at all nodes and a log likelihood value of -7634.49.

Rainbow trout β_2 -AR gene expression was examined using the RNase Protection Assay (RPA) and a gene specific probe of 95 bases that corresponded to the third intracellular loop region of the receptor. Hybridization of the RbT β_2 -AR probe to trout

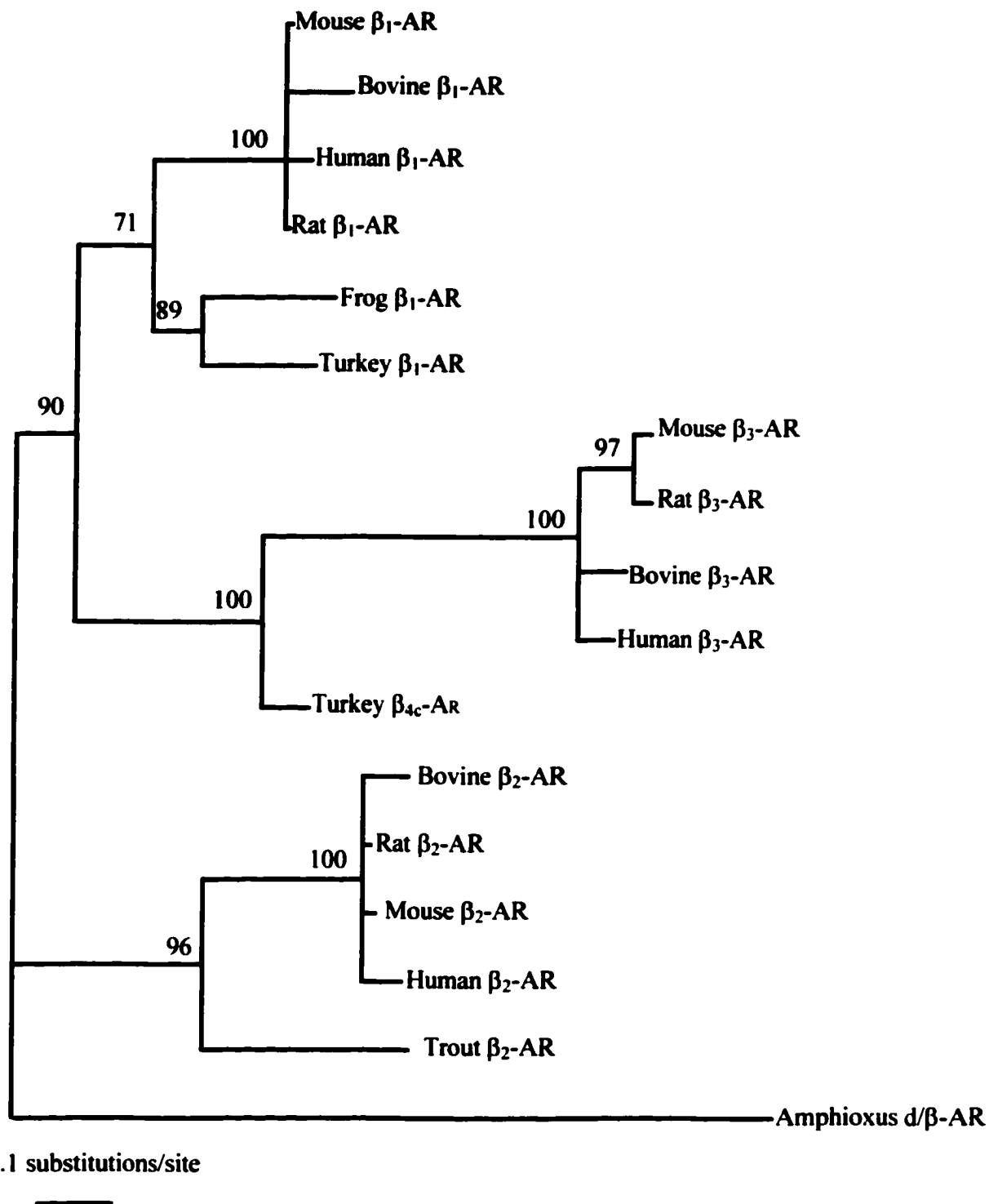


Figure 2.2 Maximum likelihood tree of selected β -AR genes, including the rainbow trout β_2 -AR, inferred from an amino acid alignment. The support values at the nodes are indicated as a percentage from 1000 puzzling steps.

tissue total RNA produced fully protected fragments in the liver, red muscle, and white muscle after 24 h of autoradiography (result not shown). After 64 hours fully protected fragments were also visible in the gills, heart, kidney and spleen (Figure 2.3). There was no evidence of RbT β_2 -AR expression in the blood. This same pattern of β_2 -AR tissue expression was reproducible in three separate individuals.

Pharmacological characterization of the RbT β_2 -AR was carried out on trout semi-purified hepatocyte membranes. ^3H -CGP was chosen over ^{125}I -iodocyanopindolol (^{125}I -CYP) as the latter has been previously shown to have a slight selectivity for the β_2 -AR whereas ^3H -CGP has been shown to have no subtype selectivity in mammalian β -AR studies (Morin *et al.*, 1992). ^3H -CGP should therefore, not bias our result towards any specific β -AR subtype. The agonists and antagonists were chosen based on previous studies that characterized β -ARs in other species and tissues (Gamperl *et al.*, 1994; García-Sáinz *et al.*, 1996; S.G. Dugan and T.W. Moon unpublished results).

^3H -CGP binding to trout hepatic β -ARs (K_d $.29 \pm 1.1$ ηM , $n=5$) was inhibited most effectively by antagonists and agonists with higher affinity for β_2 -ARs versus β_1 -ARs. Cold CGP inhibited ^3H -CGP binding the most effectively, with a K_i value of 4.3 ± 1.7 nM ($n=5$). Bromoacetyl alprenolol menthane (BAAM), an antagonist with higher affinity for β_2 - than β_1 -ARs, and the selective β_2 -antagonist ICI 118,551 (ICI) both inhibited ^3H -CGP binding much more effectively than the selective β_1 -antagonist atenolol (ATL) (Figure 2.4). The K_i values for BAAM and ICI inhibition were 0.71 ± 0.09 μM ($n=5$) and 1.3 ± 0.5 μM ($n=5$), respectively. Atenolol produced less than 50% inhibition, therefore no K_i value can be estimated. Similarly, the selective β_2 -agonists

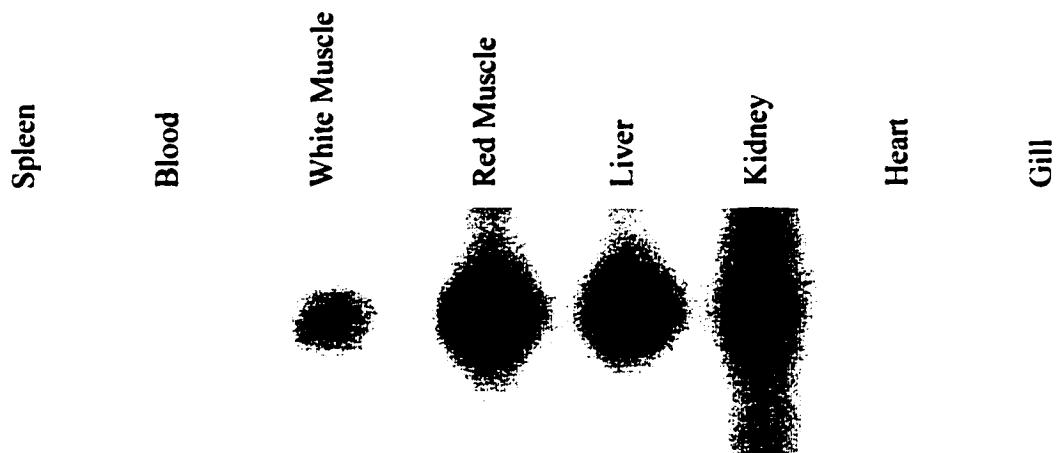


Figure 2.3 RNase protection assay showing the expression pattern of the β_2 -AR gene in eight trout tissues. Following 64 hours of autoradiography fully protected fragments (95 bases) are visible in seven of the eight tissues examined. Band intensities indicate the highest levels of RbT β_2 -AR gene expression in the liver and red muscle followed by white muscle, spleen and kidney, no band is visible in the blood. The same pattern of expression was reproducible in three separate experiments using tissue RNA from different trout.

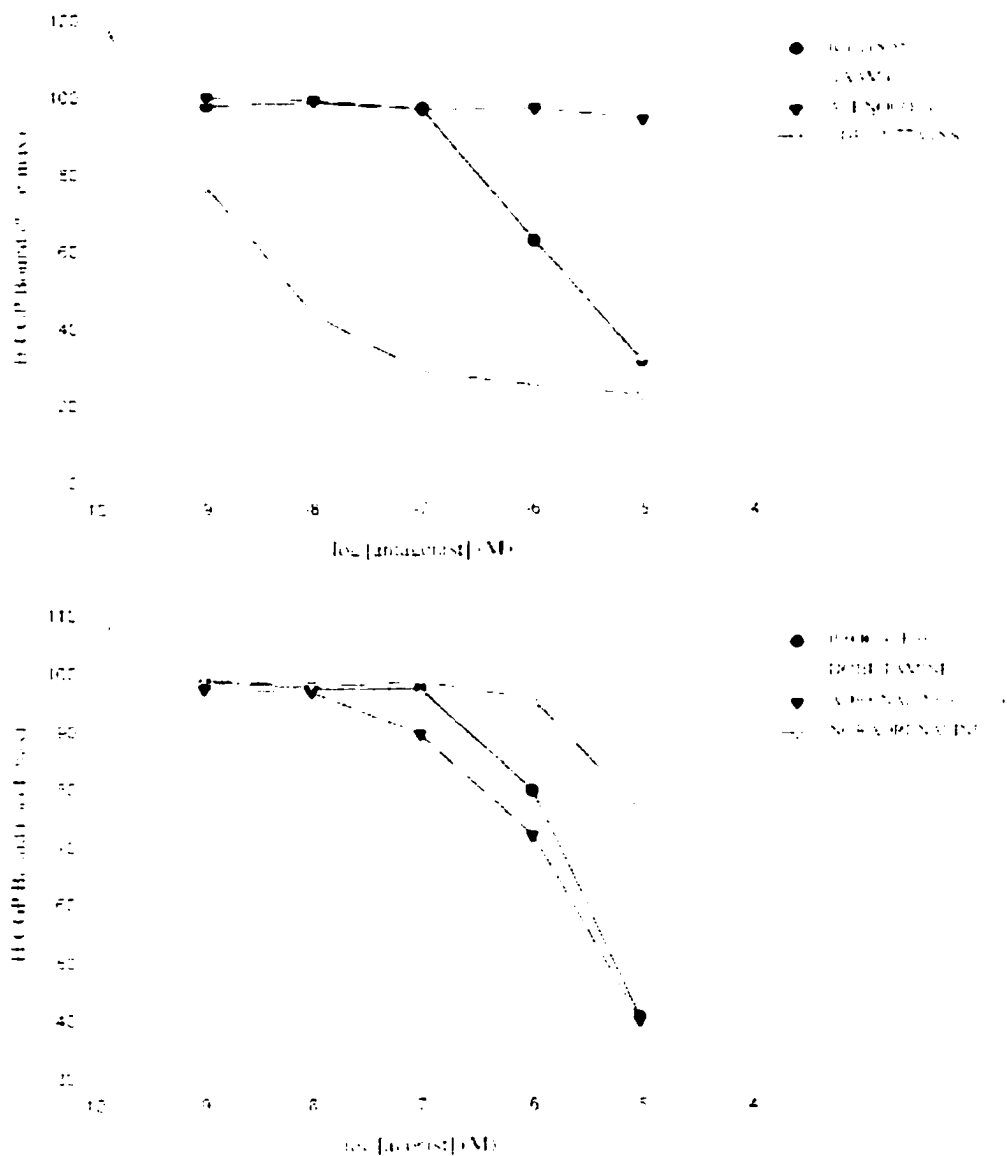


Figure 2.4(A) Competitive displacement of $^3\text{H-CGP}$ binding to trout hepatocyte membranes by the selective β_2 -antagonists ICI and BAAM and the selective β_1 -antagonist ATL. Semi-purified RbT liver membranes were incubated in the presence of a saturating concentration of $^3\text{H-CGP}$ alone or in combination with increasing concentrations of the antagonists, ICI, BAAM or ATL. **(B)** Competitive displacement of $^3\text{H-CGP}$ binding to trout hepatocyte membranes by the selective β_2 -agonists PROC and Adr and the selective β_1 -agonists DOB and Nadr. Semi-purified RbT liver membranes were incubated in the presence of a saturating concentration of $^3\text{H-CGP}$ alone or in combination with increasing concentrations of the agonists, PROC, ADR, DOB or NADR. All points represent mean values based on $n = 5$.

procatamol (PROC) and adrenaline (ADR) displaced ^3H -CGP more effectively than the selective β_1 -agonists dobutamine (DOB) and noradrenaline (NADR) (Figure 2.4). K_i values for PROC and Adr were $3.3 \pm 0.6 \mu\text{M}$ (n=5) and $3.0 \pm 1.0 \mu\text{M}$ (n=5), respectively. Neither DOB nor Nadr achieved 50% inhibition.

Discussion

The β -AR gene-family of mammals has been well characterized at the molecular, pharmacological and functional levels (Rohrer, 1998). These studies have revealed complex tissue specific gene expression patterns and receptor regulation (Strosberg, 1993). β -adrenoceptors from tissues of several fish species have been characterized by binding assays that employ agonists and antagonists shown to have specific affinities for mammalian β -AR subtypes. Identification of fish β -AR subtypes based solely on the use of pharmacological agents with specific affinity for mammalian β -AR subtypes may be problematic due to the considerable evolutionary divergence between fishes and mammals. The classification of the sequence reported here as being a RbT β_2 -AR subtype provides molecular biological support for this previous pharmacological evidence.

At the molecular level, the RbT β_2 -AR amino acid sequence shows a high degree of overall sequence conservation with mammalian β_2 -ARs. The highest level of sequence conservation occurs within the transmembrane domain regions while the amino and carboxyl terminal tails have the lowest levels of sequence conservation (Figure 2.1). Phylogenetic analysis using a maximum likelihood method indicates that the RbT β -AR sequence is most closely related to mammalian β_2 -ARs relative to other vertebrate β -AR subtypes (Figure 2.2). Analyses using other phylogenetic methods, such as maximum parsimony and neighbor joining, produce the same tree topology (result not shown). The basal position of the RbT β_2 -AR sequence in the tree relative to the mammalian β_2 -AR sequences is expected given the evolutionary relationship between fish and mammals. The phylogenetic analysis provide evidence that the sequence reported here is a RbT β_2

subtype and this is further supported by competitive binding assays on trout hepatic membranes (Figure 2.4).

Our RNase protection assays (RPA) demonstrate that the RbT β_2 -AR gene is expressed in several tissues, including liver (Figure 2.3). Subsequent biochemical characterization of trout hepatic β -ARs by competitive binding experiments shows that ^3H -CGP is most effectively displaced by β_2 -specific rather than β_1 - specific AR agonists and antagonists supporting the presence of a membrane localized β_2 -AR (Figure 2.4). This result is in agreement with a previous pharmacological characterization of rainbow trout hepatic β -ARs (Reid *et al.*, 1992) and with previous Scatchard analysis of ^3H -CGP binding to purified hepatic membranes from rainbow trout that supported the presence of a single class of β -AR binding sites (Fabbri *et al.*, 1995; Dugan and Moon, 1998). The presence of a β_2 -AR subtype by binding studies in liver membranes has been shown in several other species including teleost (black bullhead, *Ameiurus melas*; American eel, *Anguilla rostrata* [Fabbri *et al.*, 2001]) and dipnoan (Australian lungfish, *Neoceratodus forsteri* [Janssens and Grigg, 1988]) fishes, amphibians (axolotl, *Ambystoma mexicanum*; toad, *Xenopus laevis* [Janssens and Grigg, 1988]) and mammals (rat, *Rat norvegicus* [Wolfe *et al.*, 1976]; dogs, *Canis familiaris* [García-Sáinz *et al.*, 1996]; and humans, *Homo sapiens* [Kawai *et al.*, 1986]). These receptors are consistently shown to be linked to changes in cAMP levels and mobilization of hepatic glycogen, two key components of the downstream signaling system of the β_2 -AR.

RNase protection assays indicate expression of a β_2 -AR gene in six other rainbow trout tissues (Figure 2.3). Expression of the RbT β_2 -AR gene in some of these tissues is consistent with previous publications that showed by pharmacology the presence of a β -

AR. This includes the rainbow trout heart (Ask *et al.*, 1981; Keen *et al.*, 1993; Gamperl *et al.*, 1998), gills (Randall and Perry, 1992), and red and white muscle (M. Lortie and T.W. Moon, unpublished results). Pharmacological evidence for β -ARs has also been shown in several of these tissues in other fish species and early branching vertebrates (Janssens and Lowrey, 1987; Janssens and Grigg, 1988; Sundin, 1995). To our knowledge the presence or absence of β -ARs has not been examined at the biochemical level in the rainbow trout kidney or spleen. The absence of RbT β_2 -AR expression in the red blood cell is conspicuous due to the well established presence of a β -AR on the membrane of trout red blood cells (Reid *et al.*, 1991; Perry and Reid, 1993). Trout red blood cell β -ARs control the activity of a Na/H⁺ exchanger (β NHE) located in the red cell plasma membrane. Stimulation of red blood cell β -ARs leads to β NHE activation that ultimately results in an increased hemoglobin/oxygen binding affinity during times of stress (Perry and Reid, 1993). Our results suggest that signaling through a β_2 -AR subtype does not control the activity of the trout red blood cell β NHE and raises an interesting question as to the subtype identity of the trout red blood cell β -AR.

Phosphorylation by protein kinase A (PKA), protein kinase C (PKC), or β -adrenergic receptor kinases (β ARK) plays a major role in regulating the β -adrenergic response (Strosberg, 1993; Sundin, 1995). Previous studies showed that phosphorylation of residues within the third intracellular loop and the cytoplasmic tail of β -ARs result in the desensitization or uncoupling of the receptor from the G-protein, sequestration of receptors to internal cellular membranes and ultimately, degradation or recycling of the receptor protein (Lomasney *et al.*, 1995; Ferguson, 2001). The impact of these events is a change in adrenergic responsiveness in the affected cells. It is interesting to note that

while the RbT β_2 -AR sequence does possess putative phosphorylation sites within the third intracellular loop and cytoplasmic tail regions, none of these potentially phosphorylatable residues are located within the G-protein binding domain of the receptor (see Figure 2.1). In the multiple sequence alignment of β -ARs used for phylogenetic analysis (result not shown), the position of phosphorylation sites within the G-protein binding domain was conserved in the β_1 - and β_2 -ARs of other vertebrate species. This lack of phosphorylation sites within the G-protein binding domain of the RbT β_2 -AR may impact the regulation of this receptor *in vivo* as these sites are reported to play a major role in the desensitization/uncoupling of mammalian β -adrenoceptors (Yaun *et al.*, 1994; Moffet *et al.*, 1996). Lack of β -AR desensitization in the trout has been shown in a previous physiological study of the myocardium from hypoxic rainbow trout (Gamperl *et al.*, 1998). This study found that hypoxic conditions, previously shown to cause down-regulation of mammalian and avian myocardial β -ARs (e.g., Rocha-Singh *et al.*, 1991), did not reduce cell surface β -ARs in the myocardium of rainbow trout (Gamperl *et al.*, 1998). The authors proposed that resistance to down-regulation in trout myocardium may be an adaptation of the β_2 -AR allowing this species to cope with routine exposure to hypoxic conditions. It must be noted that the RbT β_2 -AR does possess phosphorylation sites in its cytoplasmic tail region; these might play a regulatory role at the level of sequestration and down-regulation. The role of carboxyl-terminal phosphorylation sites in mammalian β_2 -AR internalization remains unclear (Ferguson, 2001) since internalization has been reported in mutant β_2 -ARs that lack carboxyl-terminal phosphorylation sites (Bouvier *et al.*, 1988; Ferguson *et al.*, 1995).

With respect to the rainbow trout β -AR gene-family, we have obtained partial sequence of two additional trout β -AR genes (data not shown) suggesting the possible existence of trout homologues to other mammalian β -AR subtypes. The presence of multiple genes for trout β -AR subtypes is consistent with previous pharmacological characterizations that showed trout hepatocytes and cardiac myocytes possess β -ARs kinetically distinct from β -ARs present on the red blood cell (Reid *et al.*, 1992; Gamperl *et al.*, 1994; Gilmour *et al.*, 1994).

The isolation and sequencing of the RbT β_2 -AR gene are initial steps toward a broader understanding of the β -adrenergic system in fish and the functioning of vertebrate β -ARs in general. Our future work will focus on several areas including the isolation and characterization of additional trout β -AR subtype genes, the effect of environmental and physiological disturbances on trout β -AR gene expression, and the physiological significance of the apparent lack of PKA phosphorylation sites within the G-protein binding domain of the RbT β_2 -AR. Broadening our knowledge of the adrenergic system from early branching vertebrates is a crucial step in understanding the evolution of the complex adrenergic system of vertebrates including mammals.

Chapter 3: Characterization of the Rainbow Trout β_{3a} - and β_{3b} -Adrenoceptor Genes

Introduction

β -Adrenoceptors (β -ARs) are heptahelical, G-protein coupled, receptors that play a pivotal role in the vertebrate stress response by transducing the cellular effects of the catecholamines adrenaline (Adr) and noradrenaline (Nadr) (Strosberg, 1993; Lomasney *et al.*, 1995; Mayor *et al.*, 1998). Extensive characterization of the mammalian β -AR gene-family indicates the presence of three distinct subtypes β_1 -, β_2 -, and β_3 -ARs; each subtype is encoded by a separate gene and displays distinct pharmacological binding properties (Strosberg, 1993; Milligan *et al.*, 1994; Rohrer, 1998). The β -AR gene-family is part of the larger catecholamine receptor gene-family that includes α_1 - and α_2 -AR subtypes (α_{1a} -, α_{1b} -, and α_{1c} -ARs and α_{2a} -, α_{2b} -, and α_{2c} -ARs) as well as dopamine receptors (Fryxell, 1995).

The typical β -AR signaling pathway in mammals involves G_s -protein mediated activation of adenylyl cyclase that leads to increased intracellular cAMP and ultimately results in increased protein kinase A (PKA) activity (Lomasney *et al.*, 1995; Kompa *et al.*, 1999). PKA-mediated phosphorylation regulates the activity of a number of tissue specific cellular enzymes. Thus through the activation of PKA Adr and Nadr are able to modulate a variety of key physiological parameters including blood glucose levels, heart rate, force of cardiac contraction, and thermogenesis in adipose tissue (Freedman *et al.*, 1995; Rohrer, 1998; Chaudhry and Granneman, 1999). The tissue specific physiological responses initiated by Adr/Nadr release assist the organism to cope with various

environmental and physiological stressors (Fabbri and Moon, 1994; Perry *et al.*, 1996; Reid *et al.*, 1998).

Previous studies of fish β -ARs indicate that many of the same physiological responses elicited by β -ARs in mammals are also modulated by typical β -AR signaling in fishes (Reid *et al.*, 1998; Fabbri *et al.*, 2001). Some β -AR mediated responses are unique to groups of fishes, for example the β -AR/sodium proton exchanger (β NHE) system present in the red blood cell of many teleost species (Perry and Reid, 1993; Perry *et al.*, 1996). The rainbow trout red blood cell β -AR/ β NHE system is well characterized physiologically and serves as a model for this teleost red blood cell system (Reid *et al.*, 1991; Perry and Reid, 1993). Activity of the β NHE is controlled by signaling through a β -AR located on the plasma membrane of the trout red cell (Thomas and Perry, 1992; Perry *et al.*, 1996). Activation of the β NHE results in the alkalization of the red cell cytoplasm leading to increases in hemoglobin/O₂ binding affinity (Nikinmaa 1992; Perry *et al.*, 1996). Extensive physiological characterization of the trout blood cell β -AR suggests that this receptor is a β_1 -AR subtype (Tetens *et al.*, 1988; Perry and McDonald, 1993); however no molecular biological data exist to support this theory.

The subtle differences between the mammalian β -AR subtypes are proposed as contributing factors in the ability of Adr and Nadr to regulate a wide variety of physiological parameters in different tissues. Differences at the level of β -AR tissue specific expression, G_s-protein coupling efficiency, ability to couple to non-G_s-protein signaling pathways, differential phosphorylation of compartmentalized PKA substrates, and susceptibility to desensitization (Emorine *et al.*, 1991; Liggett and Raymond, 1993; Strosberg, 1993; Chaudhry and Granneman, 1999; Port and Bristow, 2001) likely

contribute to the flexibility/specificity of the adrenergic stress response in mammals. Previous studies of fish indicate the presence of multiple, pharmacologically distinct β -ARs in fish tissues (Gamperl *et al.*, 1994; Gilmour *et al.*, 1994; Katayama *et al.*, 1999). Thus the possibility that differences between fish β -AR subtypes could contribute to a complex β -adrenergic response in fish seems likely. A definitive answer to this question requires molecular biological characterization of the fish β -AR gene-family. However, the β -AR system of early branching tetrapods and fish remains poorly characterized at the molecular level.

We report the primary sequence, tissue expression patterns, and phylogenetic relationship of two new rainbow trout (*Oncorhynchus mykiss*) (RbT) β -ARs. Phylogenetic analysis indicates that these two trout β -ARs are homologous to the mammalian β_3 -AR thus we call these receptors RbT β_{3a} - and β_{3b} -ARs. The cloning of these two new trout β -ARs together with the recently reported cloning of a putative RbT β_2 -AR (Nickerson *et al.*, 2001) brings the total number of RbT β -AR genes to three. Molecular characterization of the RbT β -AR gene-family reveals differences at the level of tissue expression patterns, regulatory phosphorylation profiles, and the primary sequence in regions of G-protein coupling between RbT β -AR subtypes. These differences provide the potential for a RbT β -AR system of comparable complexity to that of its mammalian counterpart. These data provide the first glimpse at the molecular foundation that underlies β -AR signaling in fish and should lead to a broader understanding of the β -AR stress response system in early branching vertebrates as well

as mammals. Furthermore, it answers specific questions concerning the subtype identity of the trout red blood cell β -AR and the size of trout β -AR gene-family.

Materials and Methods

Animals. Rainbow trout, *Oncorhynchus mykiss* Walbaum were obtained from Linwood Acres Trout Farm (Campellcroft, ON). Fish were acclimated at least six weeks in fiberglass tanks containing 1275 L of aerated dechloraminated City of Ottawa tap water at 12°C with a 12L:12D photoperiod. Fish were fed commercial trout pellets (Purina trout chow) five times a week.

Isolation of RNA. Prior to collection of tissues trout were perfused through the heart with 1 L of saline (0.9% NaCl) to flush blood from the tissues. Total cellular RNA was isolated from fresh tissues of the rainbow trout using Trizol reagent (GibcoBRL). RNA concentrations and quality were verified using spectrophotometry and gel electrophoresis. Aliquots of RNA to be used in RT-PCR or RNase Protection Assays were treated with DNase I DNA free™ (Ambion Austin, TX, USA) before use.

Amplification of Rainbow Trout β_{3a} - and β_{3b} -AR cDNA. An initial set of trout β_{3a} - and β_{3b} -AR clones spanning the first to sixth transmembrane domains (approximately 750 bps), were amplified using a nested RT-PCR strategy. Oligo-p(dT)₁₅ primed cDNA was synthesized using the 1st Strand cDNA Synthesis Kit for RT-PCR (Roche Molecular Biologicals). A preliminary round of PCR amplification was performed using degenerate primers AdrUni 5' and AdrUni 3' (Table 3.1), followed by a second round of amplification using nested degenerate primers BetaUni 5' and BetaUni 3' (Table 3.1). The sequence of these clones was then used to design gene specific primers for 5' and 3' RACE of both trout β_{3a} - and β_{3b} -ARs.

RACE PCR. The 5' and 3' RACE System for Rapid Amplification of cDNA Ends, Version 2 (GibcoBRL) was used to amplify the 5' and 3' ends of the trout β_{3a} - and

Table 3.1 Primers used for amplification of various fragments of the rainbow trout β_{3a} - and β_{3b} -ARs.

	5' primers	Pos.	3' primers	Pos.	Anneal. Temp. °C
AdrUni	^{5'} YTTGGVYVYHTIDSINTIGAYMGITA ^{3'}	18-27	^{5'} RTADATAXIGGRTTIADISHISWRTT ^{3'}	1101-1075 ^{β3a} 1095-1069 ^{β3b}	45
BetaUni	^{5'} GGIAAVBTIVITGTXA ^{3'}	192-209	^{5'} CCARCAIARISWRRAAIRYICCCATDAT ^{3'}	981-955 ^{β1a} 975-949 ^{β1b}	45
^{5'} RACE β_{3a} GSP1			^{5'} GACACACAGAGCATGTCTACGGAG ^{3'}	396-375	25
^{5'} RACE β_{3a} GSP2			^{5'} TGCCAGTTCCCTGTCAGCAGCATATGG ^{3'}	344-320	48
^{5'} RACE β_{3a} GSP3			^{5'} CATGATGAAGATGTTAGTTATA ^{3'}	264-243	48
^{5'} RACE β_{3b} GSP1			^{5'} GACACACAGAGCACAACACTACAGAG ^{3'}	396-375	25
^{5'} RACE β_{3b} GSP2			^{5'} TGCCAGTTCCCCGTAACCACATGG ^{3'}	344-320	50
^{5'} RACE β_{3b} GSP3			^{5'} CATGATGAAAATGTTAGTCATA ^{3'}	264-243	45
^{3'} RACE β_{3a} GSP4	^{5'} GAGTGAGTGGCCAGTCCCCC ^{3'}	771-790			50
^{3'} RACE β_{3a} GSP5	^{5'} CTGAGCCACCACAGTAACAACATCC ^{3'}	802-826			50
^{3'} RACE β_{3b} GSP4	^{5'} CACAGTTGCAACATTTTGCCC ^{3'}	811-831			50
^{3'} RACE β_{3b} GSP5	^{5'} AGTGAATGGCCAGCGCCGACCG ^{3'}	772-792			60
β_{3a} RPA probe	^{5'} ATTAGGTGACACTATAGAAGVGGAGCC ACCACAGTAACAAC ^{3'}	804-822	^{5'} TAATACGACTCACTATAGGGAGACAGAG GSARAAGRYGCCCAT ^{3'}	958-1078	50
β_{3b} RPA probe	^{5'} ATTAGGTGACACTATAGAAGVGGAGACA AGACCCGGCTGGCC ^{3'}	747-765	^{5'} TAATACGACTCACTATAGGGAGACAGAG GSARAAGRYGCCCAT ^{3'}	952-972	50
β_{3b} CDS	^{5'} TAGGAGACAGACATACATGTTTGA ^{3'}		^{5'} ATTCACGAACTGTGTGTTGCC ^{3'}	3' UTR	50

Sequences are given according to the IUPAC code.

β_{3b} -AR cDNAs. In the 5' RACE protocol, trout β_{3a} - and β_{3b} -AR gene specific primers β_{3a} GSP1 and β_{3b} GSP1 (Table 3.1) were used to prime cDNA synthesis of trout β_{3a} - or β_{3b} -AR separately. These cDNAs were then used as template in an initial round of PCR amplification using a second set of primers specific for either trout β_{3a} -AR (β_{3a} GSP2) or β_{3b} -AR (β_{3b} GSP2) (Table 3.1) and the 5' amplification primer provided with the kit. A 5 μ L aliquot of the initial PCR amplification was then used as a template for a second round of PCR using nested primers specific for either trout β_{3a} -AR (β_{3a} GSP3) or β_{3b} -AR (β_{3b} GSP3) (Table 3.1) and the abridged universal amplification primer (AUAP) provided with the kit.

Synthesis of cDNA for 3' RACE was primed with the 3' amplification primer provided in the kit. A first round of PCR amplification was performed using trout β_{3a} - or β_{3b} -AR gene specific primers, β_{3a} GSP4 or β_{3b} GSP4, respectively (Table 3.1) and AUAP. A second round of semi-nested PCR amplification using trout β_{3a} - or β_{3b} -AR gene specific primers β_{3a} GSP5 or β_{3b} GSP5 (Table 3.1) and AUAP was then carried out.

The complete coding region of the trout β_{3b} -AR was PCR cloned using non-degenerate primers. RbT β_{3b} CDS, designed from the sequence of the β_{3b} -AR 5' and 3' RACE clones.

All PCR amplifications described above used the following regimen of denaturing, annealing and extension: 1 x 2 min at 94°C, 30 x (30 sec at 94°C, 30 sec at 45°C-60°C, 1 min at 72°C), and 1 x 10 min at 72°C. Annealing temperatures varied from 45°C to 60°C depending on the primer sets being used (Table 3.1).

Sequence analyses. The rainbow trout β_{3a} - and β_{3b} -AR amino acid sequences were aligned with Genbank sequences of various β -AR subtypes from selected organisms

(Table 3.2) using default settings in CLUSTAL W version 1.8 (Thompson *et al.*, 1994). Maximum likelihood analysis was performed using PROML version 3.6a2.1 (Felsenstein, 1993). The following program settings were used: search for best tree, JTT model of amino acid substitution, one category of site, gamma + invariant rate variation among sites, rates at adjacent sites are independent, no weighting of sites, rough analysis, global rearrangements, use sequence input order, coefficient of variation of substitution rate among sites = 1.155, number of categories = 6, and fraction of invariant sites = 0.137. Note that the values for the coefficient of variation and fraction of invariant sites were obtained from PUZZLE analysis of the alignment (Strimmer and von Haeseler, 1996). The amphioxus dopamine receptor/ β -AR sequence was used as outgroup in the maximum likelihood analysis. The trout β_{3a} - and β_{3b} -AR sequences were analyzed for the presence of gene conversion events using GENECONV version 1.70 (Sawyer, 1999). Since GENECONV analyses work best with more than two sequences this analysis was performed on a data set composed of the trout β_{3a} -, trout β_{3b} - and puffer fish β_3 -AR sequences (Drouin, 2002). The analysis was performed using a g scale of 2 to allow for some mismatches in the converted regions (Sawyer, 1999). Putative phosphorylation sites were identified by NetPhos, a neural network program that predicts potential phosphorylation sites based on sequence and structure (Blom *et al.*, 1999).

RNase Protection Assay. The expression patterns of the rainbow trout β_{3a} - and β_{3b} -AR genes were determined using the RNase Protection Assay, RPAIII™ (Ambion). Eight tissues were assessed; gill, heart, kidney, liver, red muscle, white muscle, blood, and spleen. The template used to synthesize probe for RNase protection assay experiments was obtained by PCR amplifying a region including the 3rd intercellular loop

Table 3.2 GenBank accession numbers of sequences used in the phylogenetic analysis.

	β_1 -AR	β_2 -AR	β_3 -AR
cat	AAF04303.1	AAF04304.1	AAF04592.1
dog	AAB93648.1	CAA64316.1	AAF08307.1
bovine	AAF21435	Q28044	P46626
human	NP000675	AAB82149	AAA35550
monkey	P47899	Q28509	AAB53939.1
mouse	1919247A	P18762	P25962
rat	S12591	NP036624	P26255
pig	AAB97525.1	AAB58713.1	AAG31168.1
trout	————	AAK94672.1	————
puffer fish	————	http://fugu.hgmp.mrc.ac.uk/	
turkey	P07700	————	P43141
frog	O42574	————	————
amphioxus	*CAA06536	————	————

* This sequence is that of the amphioxus dopamine receptor/ β -AR.

from a plasmid clone of the trout β_{3a} - or β_{3b} -AR. The gene specific probes corresponded to nucleotides 804 to 958 for RbT β_{3a} -AR and 747 to 952 for RbT β_{3b} -AR. The primers used to amplify probe template incorporated the promoter sequences for T7 and SP6 RNA polymerases (Table 3.1) so that antisense or sense RNA probes could be transcribed. Radiolabeled antisense RNA probes were transcribed using MAXIscript™ (Ambion) with T7 RNA polymerase and ^{32}P -UTP (Amersham Pharmacia). Full-length probe was isolated from a denaturing 4% polyacrylamide, 8 M urea gel. Approximately 4.2×10^4 cpm of probe was hybridized to 20 μg of total RNA for approximately 16 h at 42°C. Nonhybridized transcripts were digested with approximately 0.4 units of RNase A and 15 units of RNase T1 at 37°C for 90 min. Protected fragments were resolved on a denaturing 6% polyacrylamide, 8 M urea gel that was dried and subjected to autoradiography.

Kinetic analysis of receptor binding. Kinetic analysis of the rainbow trout β_{3b} -AR was carried out in intact trout red blood cells (RBCs) using the β_1 -/ β_2 -AR non-selective hydrophilic antagonist ((-)-4-(3-t-butylamino-2-hydroxypropoxy-[5,7- ^3H]benzimidazol-2-one)[^3H]CGP-12177 ([^3H]CGP; Amersham, specific activity 46.0 Ci-mmol $^{-1}$). Whole blood was diluted 10-fold in Hanks' buffered saline (in mM: 136.7 NaCl, 5.4 KCl, 0.8 MgSO $_4$, 0.33 NaH $_2$ PO $_4$, 0.44 KH $_2$ PO $_4$, 5.0 Hepes, 5.0 Hepes-Na, 1.0 NaHCO $_3$, and 0.06 L-ascorbic acid, pH 7.6). Fifty μL of cells were incubated for 45 min at room temperature in a final volume of 150 μL in the presence of a saturating concentration of [^3H]CGP (approximately 5 nM) alone or with increasing concentrations of selective and non-selective agonists and antagonists. With the exception of CL 316,243, a gift from Dr. Jean Himms-Hagen (Department of Biochemistry, Microbiology

and Immunology, Faculty of Medicine, University of Ottawa) all agonists and antagonists used were purchased from Sigma (St.Louis MO). Incubations were carried out in the dark and aluminum foil was used to cover the ligand containing microcentrifuge tubes in order to prevent photodegradation. Assays were terminated with 4 washes of ice cold 0.9% NaCl with a cell membrane harvester (Brandel 24R). Glass fibre filters (#32, Schleicher and Schuell) were placed in scintillation vials containing 4 mL scintillation cocktail (Safety-Solve; RPI, Mount Prospect, IL, USA). Radioactivity was determined using a Packard 2500TR liquid scintillation counter following 24 h of incubation in the dark.

Results

Maximum likelihood analysis of aligned vertebrate β -AR amino acid sequences including RbT β_{3a} - and β_{3b} -ARs produced a tree with three major groups corresponding to the three pharmacologically defined β -AR subtypes and placed the trout and puffer fish β_3 -ARs at the base of the mammalian/avian β_3 -AR group with strong bootstrap support (97%) (Figure 3.1). Phylogenetic analysis also indicated that vertebrate β_1 - and β_3 -AR subtypes are more closely related to one another than either is to the β_2 -AR subtype, although bootstrap support for this relationship is weak (52%).

The rainbow trout β_{3a} - and β_{3b} -AR genes encode proteins of 429 and 477 amino acids, respectively, and show the characteristic seven transmembrane domain secondary structure of receptors that couple to second messenger pathways through G-proteins. Comparison of the RbT β_{3a} - and β_{3b} -AR sequences reveals a high degree of sequence conservation at both the amino acid and nucleotide levels and the presence of an additional 48 amino acids in the cytoplasmic tail of the RbT β_{3b} -AR relative to RbT β_{3a} -AR (Figure 3.2 and 3.3). In addition a gene conversion event at the 5' end of the RbT β_{3a} - and β_{3b} -AR genes was detected using GENECONV (Sawyer, 1999). This conversion event is situated between bases 1 and 245, and is strongly supported ($P = 0.01269$). This region of 245 bases contains only 4 substitutions (1.6% substitution) whereas the remaining 1039 bases contain 108 substitutions (10.4% substitution) (Figure 3.3).

In a multiple alignment of β -ARs amino acid sequences (Appendix 3.1), the trout β_3 -ARs showed the highest degree of sequence identity with one another (84%). The RbT β_{3a} -AR amino acid sequence has an average identity of 54.6%, 51.2%, and 52.8% to

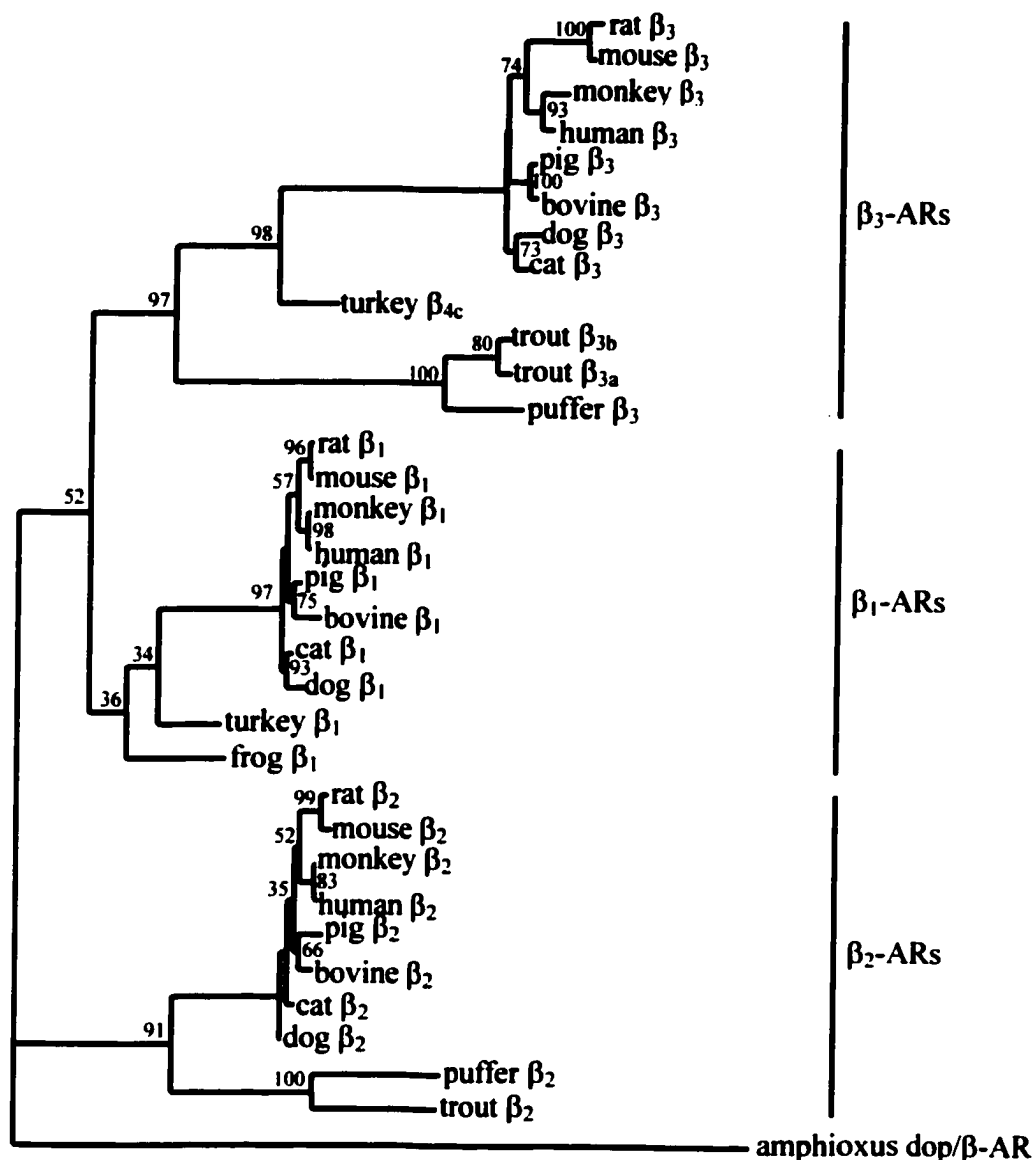


Figure 3.1 Phylogenetic relationships of vertebrate β -AR subtypes inferred from maximum likelihood analysis of β -AR amino acid sequence alignment. The three major groups correspond to one of the pharmacologically distinct β -AR subtypes. Horizontal branch lengths are scaled to represent the relative number of amino acid substitutions occurring along a branch and numbers at the nodes represent percent bootstrap support. Nodes without numbers represent relationships which were not supported by bootstrap analysis. The amphioxus dopamine/ β -AR sequence was used as an outgroup.

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trout $\beta_{3a}$  MHEKSILPLCMSFLMKMDFNLSSIVLYAVNTTPTVPHNNTGPHYHPAAGPWSLLLLV IIM 60
trout $\beta_{3b}$  MHEKSILPLCMSFLMKMDFNLSSIVLYAVNTTPTVPHNNTGPHYHPAAGPWSLLLLV IIM 60

trout $\beta_{3a}$  TIVGNLLVIAIARTSQLOTITNIFIMSLCADLIMGVLVVPLGATIVTGNWQLSDTS 120
trout $\beta_{3b}$  TIVGNLLVIAIARTSQLOTITNIFIMSLCADLIMGVLVVPLGATIVTGNWQLSDTS 120

trout $\beta_{3a}$  CELWTSVDILCVTASIEITLCVIAVDRIYIAITRPLRHKVLNKRARLIVCVVWIVSALIS 180
trout $\beta_{3b}$  CELWTSVDILCVTASIEITLCVIAVDRIYIAITRPLRHKVLNKRARLIVCVVWIVSALIS 180

trout $\beta_{3a}$  FVPIMNQYWRAEEDPEAEFCYQDPACCFEINRTFAISSVVSFYIPLLIMIFVYAKVFL 240
trout $\beta_{3b}$  FVPIMNQYWRAEEDPEAEFCYQDPACCFEINRTFAISSVVSFYIPLLIMIFVYAKVFL 240

trout $\beta_{3a}$  IATRQVQLIDKIRLRFQSECPVPTQQLSHSNILPLGYGGSCSTSSARKRSSRRR 300
trout $\beta_{3b}$  IATRQVQLIDKIRLRFQSECPVPTQQLSHSNILPLGYGGSCSTSSARKRSSRRR 298

trout $\beta_{3a}$  PSRLTVIKEHKALKTTLGIIMGTFTLCWLPFFVANIINVFDNRNPEGEIFRLLNWLGYVNS 360
trout $\beta_{3b}$  PSRLTVIKEHKALKTTLGIIMGTFTLCWLPFFVANIINVFDNRNPEGEIFRLLNWLGYINS 358

trout $\beta_{3a}$  GLNPIIYCRSEEFRTAFKNLLGCPWFSTPRNTIYKEERTICPCFLGSAEAGAAASFQK 418
trout $\beta_{3b}$  GLNPIIYCRSEEFRTAFKNLLGCPWFSTPRNTIYKEERTICPCFLGSAEAGAAASFQK 417

trout $\beta_{3a}$  PPAAATPEG* 427
trout $\beta_{3b}$  PPAAARPEGDESLASSQSSDKSEEPSGPPHFNGNTQFSEFSKPENELNWTGEKLTFTTD 477

trout $\beta_{3b}$  AEIGMAEMRLHKGKPKEMVLVNMGKED* 504

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Figure 3.2 Alignment of RbT β_{3a} - and β_{3b} -AR amino acid sequences. Indels and amino acid substitutions occurring between RbT β_{3a} - and β_{3b} -ARs are indicated by gray highlight and transmembrane domains are indicated by rectangles.

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trout  $\beta_1$ . ATGCATGAAAATCAATTTTACCGTTTGGCATGCTTTTCTTATGAAAGATGATTTTAACTTGAGTAGATATAGCTGCTTACGGGGTCAACACCACCTGTGA 100
trout  $\beta_1$ . ATGCATGAAAATCAATTTTACCGTTTGGCATGCTTTTCTTATGAAAGATGATTTTAACTTGAGTAGATATAGCTGCTTACGGGGTCAACACCACCTGTGA 100
trout  $\beta_1$ . CCCCCCTTTCAGAACATAFCAGGACCCTACACATCCTGGACGGGGACCCTGGAGTTTACTATTGCTGGTGCATCATATCATGACCAATCATGATGACCAACCT 200
trout  $\beta_1$ . CCCCCCTTTCAGAACATAFCAGGACCCTACACATCCTGGACGGGGACCCTGGAGTTTACTATTGCTGGTGCATCATATCATGACCAACCT 200
trout  $\beta_1$ . GCTGTGCATCATGCTGCTATATAGCAGCAGCACATCCAGTTACAGACATATGACTAAACATTTTCATCATATGCTGGCTGTGCTTACCTGCATCATGAGGGGCTCTG 300
trout  $\beta_1$ . GCTGTGCATCATGCTGCTATATAGCAGCAGCACATCCAGTTACAGACATATGACTAAACATTTTCATCATATGCTGGCTGTGCTTACCTGCATCATGAGGGGCTCTG 300
trout  $\beta_1$ . GTGTGCTCTCTGGGGGGCCACCATTGTGCTTACAGGAGAACTGGCAGCTTGGAGCTCTGGACCTCTGAGATGTGCTCTGTGTCAACGG 400
trout  $\beta_1$ . GTGTGCTCTCTGGGGGGCCACCATTGTGCTTACAGGAGAACTGGCAGCTTGGAGCTCTGGACCTCTGAGATGTGCTCTGTGTCAACGG 400
trout  $\beta_1$ . CTAGCATTGAGACTCTCTTGGCCGTGATGGCAGTGGACCCTATATTTGGCATATTTGGCATTAATGCAAGGCCCCCTCAGGCACAAAGTTCTGCTCAATAAAGTGGCCGCCGACT 500
trout  $\beta_1$ . CTAGCATTGAGACTCTCTTGGCCGTGATGGCAGTGGACCCTATATTTGGCATATTTGGCATTAATGCAAGGCCCCCTCAGGCACAAAGTTCTGCTCAATAAAGTGGCCGCCGACT 500
trout  $\beta_1$ . AATAGTGTGTGGTGTGGATTGTGTCTGCTCTGATATCTTTTGGTGGCCATCATGAACCAAGCTTTGGCGCAGAGATGAGGACCCGGAGGCATGCTGTGG 600
trout  $\beta_1$ . AATAGTGTGTGGTGTGGATTGTGTCTGCTCTGATATCTTTTGGTGGCCATCATGAACCAAGCTTTGGCGCAGAGATGAGGACCCGGAGGCATGCTGTGG 600
trout  $\beta_1$ . TATCAGGACCCCTGCCCTGCTGTACTTTTGTGATTAACCGCACCTTTTGGCATTATATCTCTGTAGTGCATTTCAATCCCTTTGCTCATATGATATTGG 700
trout  $\beta_1$ . TATCAGGACCCCTGCCCTGCTGTACTTTTGTGATTAACCGCACCTTTTGGCATTATATCTCTGTAGTGCATTTCAATCCCTTTGCTCATATGATATTGG 700
trout  $\beta_1$ . TCTATGGCAAAAGTCTTCCCTATAGCCACACAGCAGATAGACGCTCATAGCAAAAGATCCCTGCGGCTTCCAGAGTGAATGGCCACGTCCCGAGAGACACACA 800
trout  $\beta_1$ . TCTATGGCAAAAGTCTTCCCTATAGCCACACAGCAGATAGACGCTCATAGCAAAAGATCCCTGCGGCTTCCAGAGTGAATGGCCACGTCCCGAGAGACACACA 800
trout  $\beta_1$ . GCTGAGCCACCACAGTAAACAGATCTGCGCCCTGGGCTATGAGGCGGCGGAGCAGAAAGCGCACCACTCAGCTCCGCAAGAGAGATCCCAAGCGGAAGG 900
trout  $\beta_1$ . GCTGAGCCACCACAGTAAACAGATCTGCGCCCTGGGCTATGAGGCGGCGGAGCAGAAAGCGCACCACTCAGCTCCGCAAGAGAGATCCCAAGCGGAAGG 900
trout  $\beta_1$ . CCTCAGCGGCTAACGGTTATCAAGSAGCAAGGCCCTCAACACCTTGGGGATTATCATGGGCACCTTCAACCCTGTGCTGGCTTCTTTGTGGCTA 1000
trout  $\beta_1$ . CCTCAGCGGCTAACGGTTATCAAGSAGCAAGGCCCTCAACACCTTGGGGATTATCATGGGCACCTTCAACCCTGTGCTGGCTTCTTTGTGGCTA 1000
trout  $\beta_1$ . ACATCATCAAGCTGTTTGGACGGAAAGTGGCCCCCGAGAGATCTTCCCGCTGCTCAACTGGCTGGGCTACTCAAAATCTGGCCCTCAACCCCATCATCTA 1100
trout  $\beta_1$ . ACATCATCAAGCTGTTTGGACGGAAAGTGGCCCCCGAGAGATCTTCCCGCTGCTCAACTGGCTGGGCTACTCAAAATCTGGCCCTCAACCCCATCATCTA 1100
trout  $\beta_1$ . CTGCAAGGAGCCCTTACTGCTGGCAGCTGCTTTCAGAAACCTACTGGGCTGGCCCTGGTFTTCCACCACGANTTAACACCTTAGGTGTCAACAGGAGAAG 1194
trout  $\beta_1$ . CTGCAAGGAGCCCTTACTGCTGGCAGCTGCTTTCAGAAACCTACTGGGCTGGCCCTGGTFTTCCACCACGANTTAACACCTTAGGTGTCAACAGGAGAAG 1194
trout  $\beta_1$ . CGGACACAGATGCCCCCTGCTTCCCTGGGTTGGGCTGAGGCAAGGGGGCGGCTCTTTTCAGAAAGCCCCCTGCTGTGGCAGCGCCTGAAAGGGTGA 1284
trout  $\beta_1$ . CGGACACACAGTCCCTGCTTCCCTGGGTTGGGCTGAGGCAAGGGGGCGGCTCTTTTCAGAAAGCCCCCTGCTGTGGCAGCGCCTGAAAGGGTGA 1284
trout  $\beta_1$ . CTAGCAGTCAAGCAGCAAGAGTGAAGGCTTCCCCAGGACCCGCCACACTTAAACGGCAACACAGCTTCACTGAAATTTCTCCAAACCAAGAAATGA 1391
trout  $\beta_1$ . ATTGAATTGGACGGGTGAAAAACTGACTTTTCACACTGACTGA 1434

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Figure 3.3 Alignment of RbT β_{3A} - and β_{3B} -AR nucleotide sequences. Transmembrane domains are indicated by rectangles, synonymous substitutions are indicated by gray highlight, and nonsynonymous substitutions and indels are indicated by stars.

other vertebrate β_1 -, β_2 -, and β_3 -ARs, respectively, while RbT β_{3b} -AR shows an average amino acid identity of 54.2%, 52.2%, and 52.0% to other vertebrate β_1 -, β_2 -, and β_3 -ARs, respectively. The highest levels of sequence conservation occurred within the transmembrane domains while the extracellular tail, third intracellular loop and cytoplasmic tail regions were the most variable (Appendix 3.1). Trout β_{3a} - and β_{3b} -ARs possess eight and six potential regulatory phosphorylation sites within their third intracellular loops, respectively (Figure 3.4). Two potentially phosphorylated residues were detected in the cytoplasmic tail region of RbT β_{3a} while eight were detected in the cytoplasmic tail of RbT β_{3b} (Figure 3.5). These potential phosphorylation sites generally do not align with those in the respective homolog.

Tissue specific expression patterns of the RbT β_{3a} - and β_{3b} -ARs were determined using RNase protection assay (RPA). RbT β_{3a} - and β_{3b} -AR gene-specific probes for RPA were designed to span the third intracellular loop, a region of sequence variability between the RbT β_{3a} - and β_{3b} -ARs (Figure 3.3). Expression of RbT β_{3a} - and β_{3b} -ARs was examined in eight tissues. RPA experiments showed high levels of β_{3a} -AR mRNA in gill and heart, low levels in red muscle, white muscle, and kidney, and no detectable expression in liver, blood, or spleen (Figure 3.6). RbT β_{3b} -AR mRNA was highly expressed only in blood; faint bands detected in the gill, heart and spleen likely represent β_{3b} -AR mRNA from blood cells contaminating these tissues (Figure 3.6).

Pharmacological characteristics of the RbT β_{3b} -AR were determined by competitive binding assays performed on intact trout red blood cells (RBC) using the non-selective β_1 -/ β_2 -AR antagonist ^3H -CGP. Association kinetics found a K_d of $5.92 \pm$

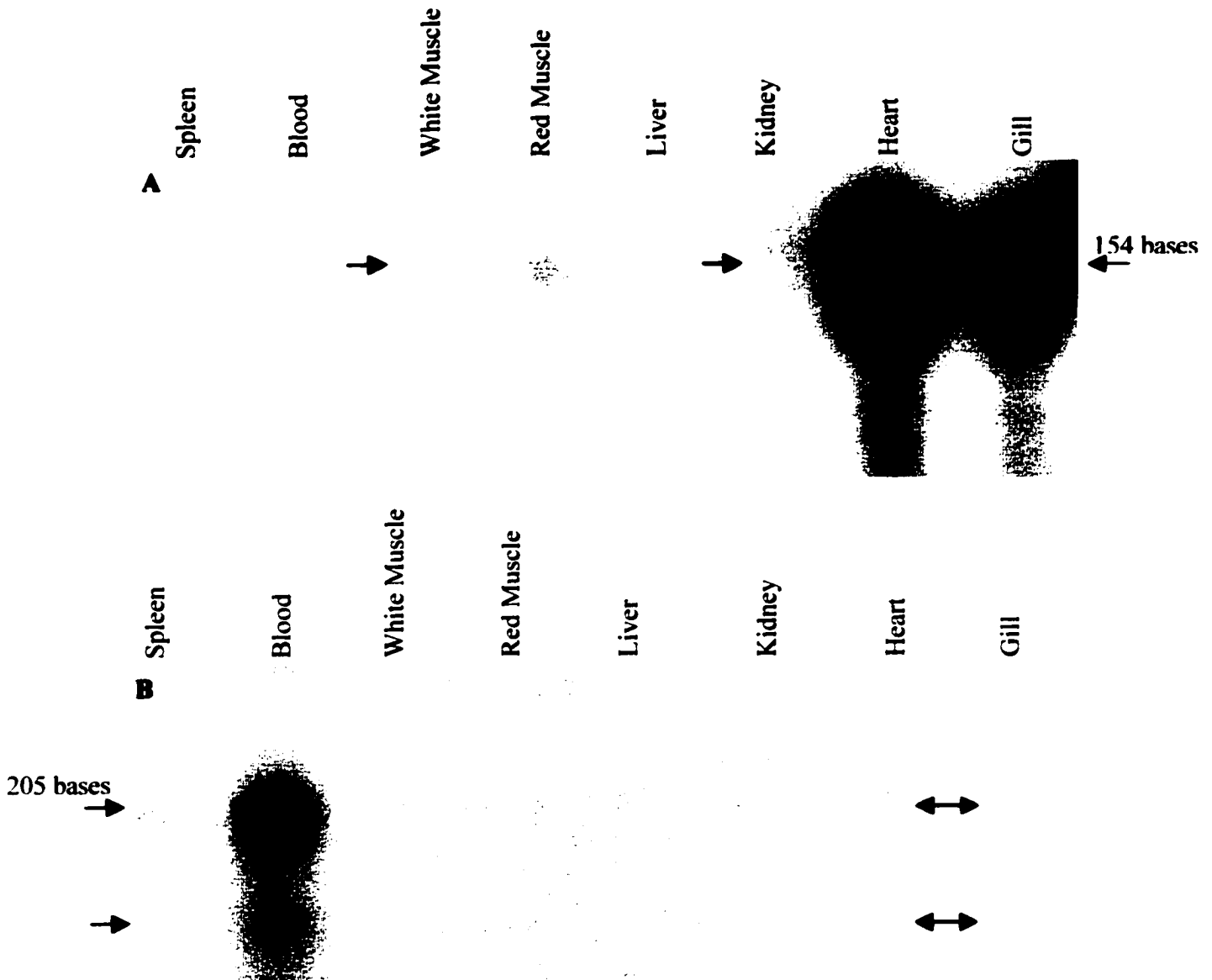


Figure 3.6 (A) RNase protection assay showing the expression pattern of the rainbow trout β_{3a} -AR gene. A β_{3a} -AR gene specific probe was hybridized to total RNA from eight different trout tissues. A fully protected fragment of 154 bases is present in gill, heart, kidney, red muscle, and white muscle. (B) RNase protection assay showing the expression pattern of the rainbow trout β_{3b} -AR gene. A β_{3b} -AR gene specific probe was hybridized to total RNA from eight different trout tissues. A fully protected fragment of 205 bases is present in blood, spleen, gill, and heart. A second smaller band caused by premature termination of probe transcription is also present in these same tissues.

1.2 nM (n=6) for ^3H -CGP binding. Among the non-selective β -AR agonists, isoproterenol inhibited ^3H -CGP binding the most effectively with a K_i of $7.14 \pm 0.78 \mu\text{M}$ (n = 5) followed by Adr and Nadr (Figure 3.7). Neither Adr nor Nadr produced greater than 50% inhibition of ^3H -CGP binding at the concentrations used; therefore, no K_i values could be estimated. Displacement of ^3H -CGP binding by β -AR subtype selective agonists indicate that only the β_2 -AR specific clenbuterol produced significant displacement of ^3H -CGP binding with a K_i of $809 \pm 168 \text{ nM}$ (n = 5) (Figure 3.7). None of the remaining β -AR selective agonists, dobutamine, procaterol, BRL 37344, or CL 316,243 achieved greater than 50% inhibition. Among the β_1/β_2 selective antagonists, the β_2 -AR selective ICI 118,552 inhibited ^3H -CGP binding most effectively with a K_i of $478 \pm 118 \text{ nM}$ (n = 4) followed by BAAM ($\beta_2 > \beta_1$; K_i $1.79 \pm 0.32 \mu\text{M}$, n = 6), and atenolol (β_1 selective; K_i = $3.05 \pm 1.18 \mu\text{M}$, n = 4) (Figure 3.8). The β -AR non-selective antagonists propranolol and nadolol both inhibited ^3H -CGP binding to trout RBCs with K_i values of $1.40 \pm 0.33 \text{ nM}$ (n = 6) and $55.3 \pm 28.7 \text{ nM}$ (n = 3), respectively (Figure 3.8).

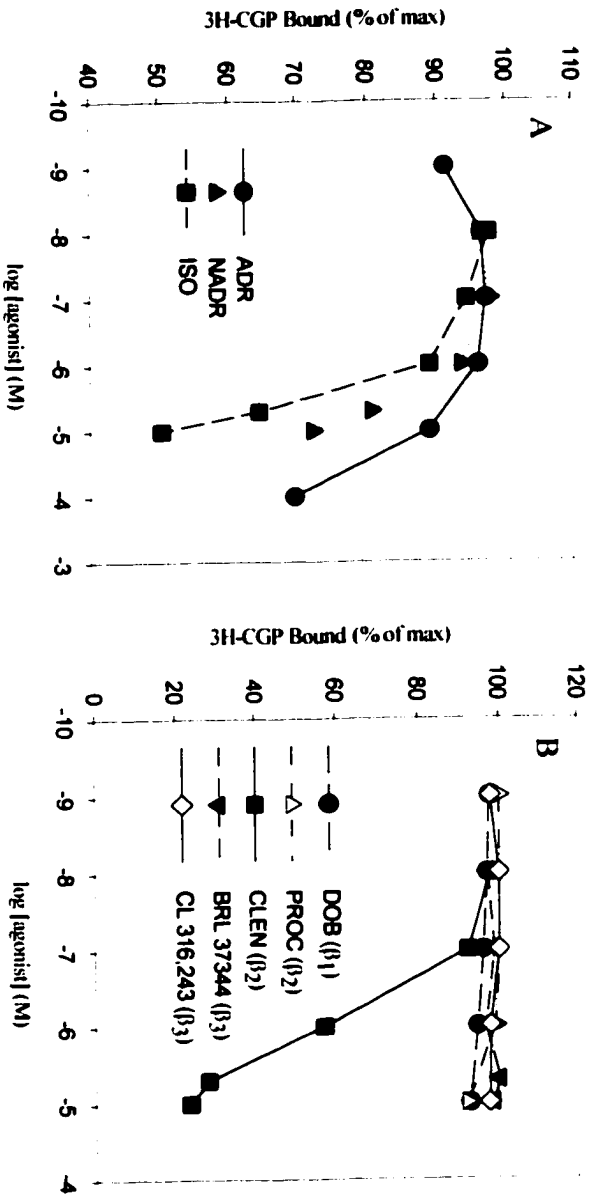


Figure 3.7 (A) Competitive displacement of ³H-CGP bound to RbT RBC β_{3b} -ARs by the endogenous hormones adrenaline (ADR), noradrenaline (NADR), and the synthetic agonist isoproterenol (ISO). (B) Competitive displacement of ³H-CGP bound to RbT RBC β_{3b} -ARs by subtype selective synthetic agonists: dobutamine (DOB), procaterol (PROC), clenbuterol (CLEN), BRL 37344, and CL 316,243.

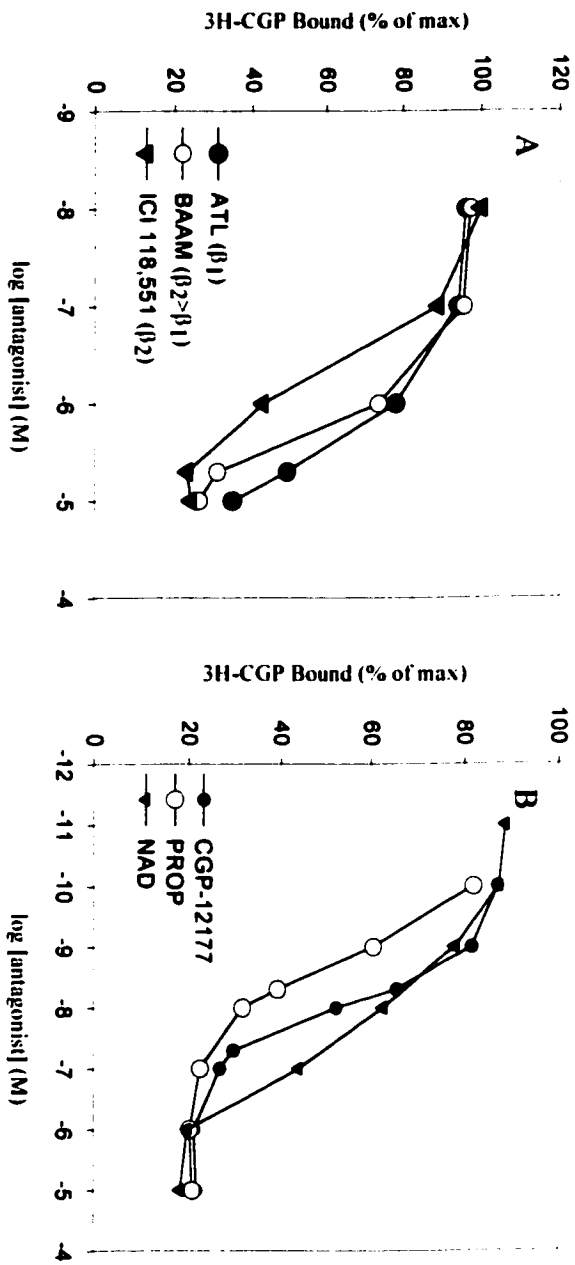


Figure 3.8 (A) Competitive displacement of ³H-CGP from RbT RBC β_1 - and β_2 -AR subtype selective synthetic antagonists: atenolol (ATL), BAAM, and ICI 118,551. (B) Competitive displacement of ³H-CGP from RbT RBC β_{3b} -ARs by non-selective synthetic antagonists CGP-121177, propranolol (Prop), and nadolol (NAD).

Discussion

Characterization of β -ARs which began over 50 years ago (Ahlquist, 1948) provides an extensive data set describing the molecular, pharmacological and physiological properties which underlie the complex and flexible β -AR signaling system in a large variety of vertebrate species (Fabri and Moon, 1994; Milligan *et al.*, 1994). To a large extent studies to characterize β -AR signaling systems have focused on mammalian species; this is due to the role β -ARs play in human disorders including cardiac disease and obesity (Davies *et al.*, 1996; Li *et al.*, 1996; Emilsson *et al.*, 1998; Vatner *et al.*, 2000; Port and Bristow, 2001). Our knowledge of β -ARs from non-mammalian vertebrates has come from relatively few studies, and this is particularly true at the molecular level where only a handful of studies describe non-mammalian β -ARs (Yarden *et al.*, 1986; Chen *et al.*, 1994; Devic *et al.*, 1997; Nickerson *et al.*, 2001). The cloning of the two putative trout β_3 -ARs reported here in addition to the previously cloned putative trout β_2 -AR (Nickerson *et al.*, 2001) indicates a trout β -AR gene-family with at least three members. The presence of three distinct RbT β -AR genes by molecular data supports previous physiology studies indicating that β -AR signaling in tissues of the trout were controlled by pharmacologically distinct β -ARs (Reid *et al.*, 1992; Gamperl, 1994; Gilmour *et al.*, 1994).

A comparison of the RbT β_{3a} - and β_{3b} -AR amino acid sequences indicates a high degree of overall identity (84%) but also reveals two regions of considerable sequence variation: the cytoplasmic tail and the third intracellular loop (Figure 3.2). When mammalian β -AR subtypes are compared with one another most of the sequence variation occurs within the extracellular and cytoplasmic tails, and the third intracellular

loop (Strosberg, 1993; Lomasney *et al.*, 1995). The apparent lack of sequence divergence between the extracellular tails of the two trout β_3 -AR sequences is explained by the presence of a gene conversion event extending from position 1 to 245 between these two genes (Figure 3.2). Gene conversion occurs between regions of DNA with similar, but not identical, sequences and is common among members of a gene-family (Cantor and Smith, 1999). Gene conversion occurs when a single stranded region from one gene invades and base pairs with a single stranded region from a second gene to produce heteroduplex DNA (Brown, 1999). Correction of mismatched base pairs within the heteroduplexed DNA results in the sequence of one gene becoming identical to that of the other gene (Figure 3.9) (Brown, 1999). N-linked glycosylation of the β -AR extracellular tail has been implicated in the proper trafficking and insertion of the receptor into the plasma membrane (Strosberg, 1993; Valdenaire and Vernier, 1997); however, the importance of this region to receptor functioning is unclear. Previous studies indicate that this region does not play a role in ligand binding (Dixon *et al.*, 1987) and furthermore this receptor region tends to be highly divergent in sequence and length between β -AR subtypes and organisms. These results suggest that the β -AR extracellular tail may not play a significant role in receptor function thus the presence of a gene conversion event between two β -ARs in this region is not likely to adversely affect receptor function and therefore would not be selected against. The presence of some non-synonymous (Figure 3.2 and 3.3) and synonymous (Figure 3.3) substitutions between the extracellular tails of the two trout β_3 -ARs rules out the possibility of cloning artifacts or differential splicing to explain the lack of sequence divergence between the first 245 bases of these two sequences.

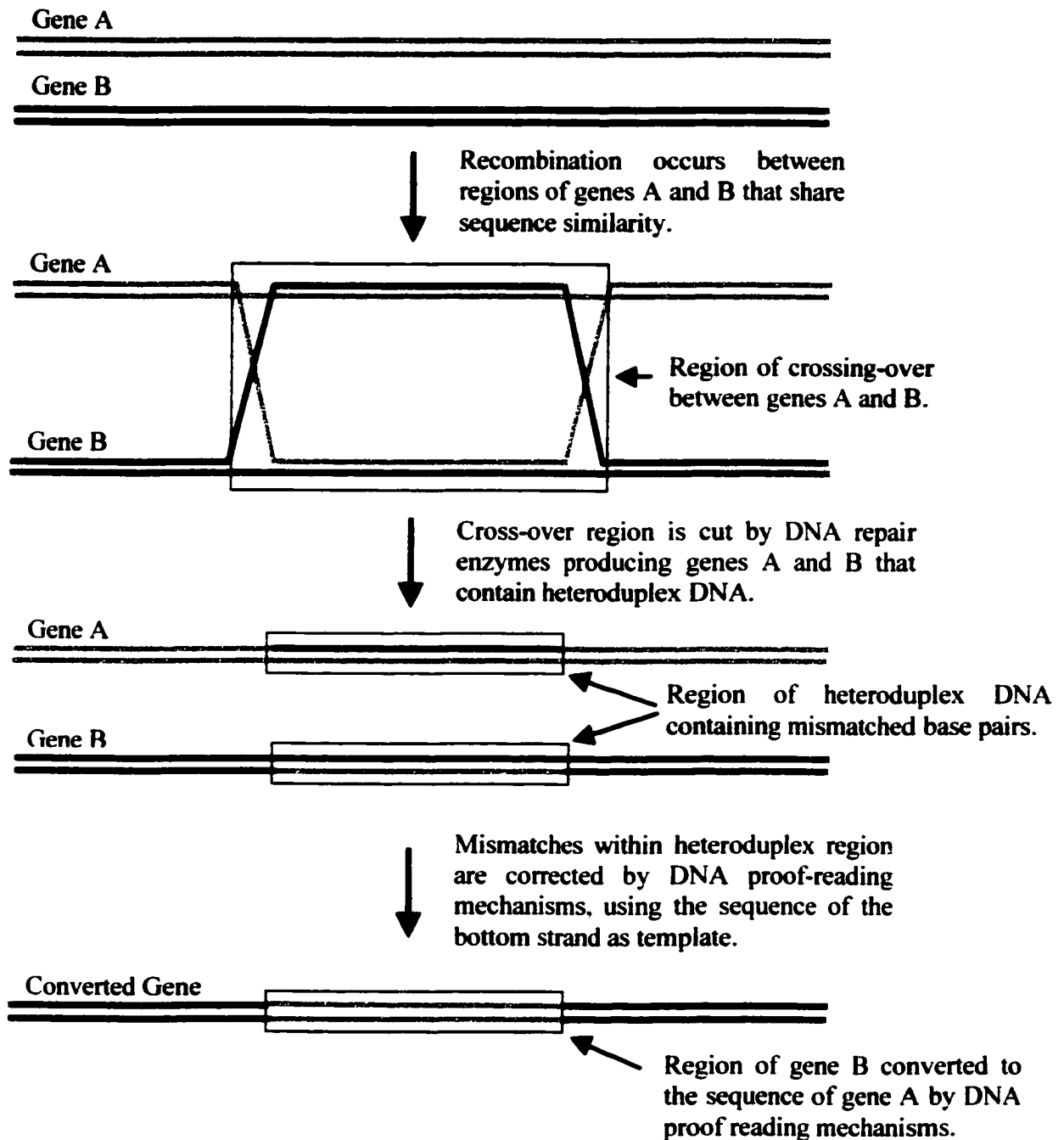


Figure 3.9 Schematic representation of gene-conversion occurring between two hypothetical genes with similar but not identical DNA sequences. In this figure gene A converts gene B. In order for gene-conversion to occur the two sequences involved must share a sufficient level of DNA sequence identity to allow recombination to occur. Note that conversion of gene B is dependent upon the use of the bottom strand as template during correction of mismatched bases by DNA proof reading mechanisms. If the top strand were used as template gene-conversion would not occur. Only the final fate of gene B is depicted.

Maximum likelihood analysis of aligned β -AR amino acid sequences (Appendix 3.1) produced a tree with three major groups, each group corresponding to one of the three pharmacological defined β -AR subtypes (Figure 3.1). Bootstrap support was generally high throughout the tree with strong support at the base of each major group. The phylogenetic analysis indicates a close relationship between β_1 - and β_3 -AR subtypes relative to β_2 -ARs. In terms of vertebrate β -AR gene-family evolution this topology implies that a gene duplication occurred previous to the split of teleosts and tetrapods giving rise to two β -AR genes. One of these genes evolved into the present day β_2 -AR, which is present in both teleost and tetrapod lineages while the second gene underwent a second duplication giving rise to β_1 - and β_3 -AR genes. If this second gene duplication occurred previous to the split of the teleost and tetrapod lineages, then both mammals and teleosts should possess β_1 - and β_3 -ARs. However, we were unable to amplify a trout β_1 -AR cDNA from any tissue and, more significantly, searches of the complete puffer fish genome failed to retrieve a puffer fish β_1 -AR. Lack of a teleost β_1 -AR homolog suggests two possibilities. Either the gene duplication giving rise to β_1 - and β_3 -ARs occurred previous to the split of teleosts and tetrapods and the β_1 -AR was subsequently lost in the teleost lineage, or the duplication event occurred after the split of teleosts and tetrapods thus teleosts never possessed a β_1 -AR. The position of the teleost β_3 -ARs as a sister group to tetrapod β_3 -ARs (Figure 3.1) supports the first hypothesis of β_1 -AR gene loss in the teleost lineage. If the second hypothesis were true, then we would expect the teleost β_3 -ARs to be a sister group to both tetrapod β_1 - and β_3 -ARs. A definitive answer concerning the existence of a β_1 -AR homolog in teleosts awaits the completion of a number of teleost genome sequencing projects currently underway. Failure to PCR

amplify a trout β_1 -AR gene using degenerate primers should not be taken as proof of its absence in this species.

With regards to the phylogenetic relationship of fish β -ARs, the basal position occupied by teleost β_2 - and β_3 -ARs relative to the mammalian β_2 - and β_3 -ARs groups (Figure 3.1) is consistent with the phylogenetic relationship of fish and mammals. The close phylogenetic relationship of the RbT β_{3a} - and β_{3b} -AR genes (Figure 3.1) may be due to the gene conversion event detected between these two sequences; however this theory can be ruled out as exclusion of the converted region from the phylogenetic analysis does not alter tree topology (result not shown). This result suggests a second hypothesis that RbT β_{3a} - and β_{3b} -AR genes show a close phylogenetic relationship because they arose from the genome duplication event that occurred near the base of the salmonid lineage (Ohno, 1970; Ferguson and Allendorf, 1991; Wolfe, 2001). The presence of only a single β_3 -AR gene in the puffer fish genome and its basal position in the phylogenetic tree relative to the two trout β_3 -ARs (Figure 3.1) also supports this hypothesis. It is interesting to note that the duplicated trout β_3 -AR gene remained functional as genetic based studies of salmonid species indicate that a large portion of duplicated genes became non-functional shortly after the genome was duplicated (Ferguson and Allendorf, 1991). We speculate that the duplicated trout β_3 -AR gene remained functional because of selective pressures to recover a third functional β -AR gene which had been previously lost in the teleost lineage. Phylogenetic analysis provides a perspective on RbT β -AR gene evolution based on primary sequence; however, information concerning potential modes of regulation, patterns of tissue

expression and pharmacology are also vital to our understanding of receptor functioning at the physiological level.

Previous studies of mammalian β -AR signaling indicate that reduced β -AR tissue response following prolonged receptor stimulation is typical of β -AR systems (Freedman *et al.*, 1995; Lomasney *et al.*, 1995; Marullo *et al.*, 1995; Kompa *et al.*, 1999; Ding *et al.*, 2000; Ferguson, 2001). The loss of tissue β -AR response, also known as desensitization, is a result of regulatory mechanisms acting at the level of the receptor. Uncoupling of receptor/G-protein interactions, sequestering of β -ARs onto internal cellular membranes, and down-regulation or loss of receptor protein all result in desensitization of the tissue β -AR response (Lomasney *et al.*, 1995; Valdenaire and Vernier, 1997; Ferguson, 2001). It is widely accepted that protein kinase A (PKA) and β -adrenergic receptor kinase (β ARK) mediated phosphorylation of β -ARs plays a central role in desensitization of the β -AR response (Lomasney *et al.*, 1995; Strosberg, 1995; McDonald and Lefkowitz, 2001). It is generally held that PKA-mediated uncoupling of receptor/G-protein interaction occurs as a result of phosphorylation of residues located in proximity to the receptor G-protein binding domains (Ferguson, 2001). β ARK-mediated phosphorylation of β -AR cytoplasmic tail residues facilitates binding of β -arrestin to the receptor which blocks receptor/G-protein interaction and also serves as a trigger for receptor sequestration (Valdenaire and Vernier, 1997; Ferguson, 2001; McDonald and Lefkowitz, 2001). A consensus pattern search for phosphorylation sites reveals that both trout β_{3a} - and β_{3b} -ARs possess several potential phosphorylation sites in the vicinity of G-protein binding domain II of the third intracellular loop located nearest the sixth transmembrane domain (Figure 3.4). In the cytoplasmic tail region, eight potential phosphorylation sites

were detected in RbT β_{3b} -AR while two sites were detected in RbT β_{3a} -AR (Figure 3.5). Our previous analysis for potential phosphorylation sites in the trout β_2 -AR revealed a deficiency in phosphorylation sites within the G-protein binding domain II of the trout β_2 -AR relative to mammalian β_2 -ARs and the presence of three cytoplasmic tail phosphorylation sites in the trout β_2 -AR (Figure 2.1). The sequence of two additional β -ARs from a second teleost, the puffer fish, were also analyzed for the presence of potential phosphorylation sites. The puffer fish β_2 -AR possesses eight potential phosphorylation sites, three are located in the third intracellular loop and five are found on the cytoplasmic tail (Figure 3.4 and 3.5). The puffer fish β_3 -AR possesses eleven potential phosphorylation sites, two are located in G-protein binding domain II and nine are found in the cytoplasmic tail (Figure 3.4 and 3.5).

A comparison of the regulatory phosphorylation profiles of trout and mammalian β -AR reveals similarities in the abundance and location of regulatory phosphorylation sites suggesting that the same PKA and β ARK-mediated mechanisms that regulate β -AR signaling in mammals also regulate β -AR signaling in trout. Mammalian β -AR subtypes show different sensitivities towards desensitization; β_1 - and β_2 -ARs are highly sensitive to desensitization while β_3 -ARs show a resistance towards desensitization (Emorine *et al.*, 1991; Ligget and Raymond, 1993; Marullo *et al.*, 1995; Chaudhry and Grannemanm, 1999). The presence of several phosphorylation sites within the third intracellular loop and cytoplasmic tail regions of mammalian β_1 - and β_2 -ARs (Figure 3.4 and 3.5) supports the evidence that these receptors are sensitive to desensitization by both PKA and β ARK-mediated pathways (Emorine *et al.*, 1991; Ligget and Raymond, 1993; Marullo *et al.*, 1995). The resistance of mammalian β_3 -ARs to desensitization has been attributed to its

truncated cytoplasmic tail relative to other β -AR subtypes and may also be due to a low abundance of phosphorylation sites (Emorine *et al.*, 1989; 1991; Liggett and Raymond 1993; Strosberg, 1993). Rainbow trout β -AR subtypes show different combinations of third intracellular loop and cytoplasmic tail phosphorylation profiles relative to their mammalian counterparts. This suggests the possibility that a particular trout β -AR subtype may be sensitive to PKA-mediated uncoupling but resistant towards β ARK-mediated sequestration and down-regulation. Within the third intracellular loop both trout β_3 -ARs have potential phosphorylation profiles similar to those of mammalian β_1 - and β_2 -ARs with an abundance of phosphorylation sites both within and in close proximity to the G-protein binding domain II (Figure 3.4); this implies that both trout β_3 -ARs are sensitive to PKA-mediated uncoupling. The RbT β_{3a} -AR possesses a truncated cytoplasmic tail with only two potential phosphorylation sites making it most similar to mammalian β_3 -ARs in this region and suggests that RbT β_{3a} -AR may be resistant to β ARK-mediated sequestration and down-regulation (Figure 3.5). On the other hand the cytoplasmic tail of RbT β_{3b} -AR has an abundance of phosphorylation sites similar to mammalian β_1 - and β_2 -ARs (Figure 3.5) indicating that RbT β_{3b} -AR may be sensitive to β ARK-mediated sequestration and down-regulation. Our previous analysis of potential phosphorylation sites in the trout β_2 -AR revealed a deficiency in phosphorylation within G-protein binding domain II of the trout β_2 -AR relative mammalian β_1 - and β_2 -ARs and the presence of three cytoplasmic tail phosphorylation sites (Figure 2.1). These results led us to propose that the trout β_2 -AR may be resistant to PKA-mediated uncoupling, while the presence of the three cytoplasmic tail phosphorylation sites indicates potential

sensitivity to β ARK-mediated sequestration and down-regulation (Nickerson *et al.*, 2001). In summary, the phosphorylation profiles of the RbT β -ARs support β_{3a} -AR sensitivity to PKA-mediated uncoupling but resistant to β ARK-mediated sequestration and down-regulation, β_{3b} -AR sensitivity to both PKA-mediated uncoupling and β ARK-mediated sequestration and down-regulation, while the β_2 -AR may be resistant to PKA-mediated uncoupling but sensitive to β ARK-mediated sequestration and down-regulation. Desensitization of the RbT RBC β -AR has been demonstrated in previous physiological studies (Gilmour *et al.*, 1994; Perry *et al.*, 1996) and in a recent study trout treated with the β_2 -AR agonist clenbuterol showed a significant reduction in hepatic β_2 -AR binding sites indicating sequestration and/or down-regulation of this receptor; however, this study did not address the issue of receptor/G-protein uncoupling (S.G. Dugan, M. Lortie and T.W. Moon, unpublished).

Tissue specific expression patterns of the RbT β_3 -AR genes were determined using the highly sensitive RNase protection assay and gene specific probes that hybridized to a region including the third intracellular loop. RbT β_{3a} -AR mRNA was present at high levels in the gill and heart and at lower levels in red muscle, white muscle, and kidney (Figure 3.6). The presence of RbT β_{3a} -AR mRNA in gill, heart red muscle, and white muscle is consistent with previous binding studies on these tissues from trout and other fish which demonstrated the presence of a β -AR (Randall and Perry, 1992; Keen *et al.*, 1993; Gamperl *et al.*, 1998; Sundin, 1995; S.G. Dugan, M. Lortie, and T.W. Moon, pers. commun.). Rainbow trout β_{3b} -AR is expressed specifically and at high levels in the blood (Figure 3.6); this result is consistent with previous physiological studies of RbT RBCs that demonstrated the presence of a β -AR (Reid *et al.*, 1991; Perry

and Reid, 1993). Expression patterns of the RbT β -AR genes reveal that trout RBCs exclusively express the β_{3b} -AR subtype. This finding is in contrast with previous physiology based studies that suggested the presence of a β_1 -AR subtype in the RbT RBC controlling the activity of the β NHE (Tetens *et al.*, 1988; Perry and McDonald, 1993). Detection of fully protected β_{3b} -AR bands in tissues other than blood was not reproducible between four separate RPA experiments using different tissue RNA samples; this leads us to conclude that the presence of faint fully protected β_{3b} -AR probe in gill and heart is likely the result of blood contamination of gill and heart tissue samples and does not reflect expression of β_{3b} -AR in these tissues (Figure 3.6).

Comparison of the tissue expression patterns of trout and mammalian β -ARs reveals parallels between these gene families. Mammalian β_1 - and β_2 -ARs have a broad tissue distribution and are known to be the dominant β -AR subtype mediating signaling in several tissues, whereas the dominance of the β_3 -AR subtype in mediating signaling is generally restricted to adipose tissue (Muzzin *et al.*, 1991; Granneman and Lahners, 1994; Lomasney *et al.*, 1995; Atgié *et al.*, 1996; McNeel and Mersmann, 1999). The RbT β_2 - and β_{3a} -ARs show broad patterns of tissue expression similar to the mammalian β_1 - and β_2 -ARs while RbT β_{3b} -AR expression is restricted to a single tissue (Figure 2.3 and 3.6).

The exclusive expression of the β_{3b} -AR subtype in RBCs of the trout shown by molecular means led us to revisit the pharmacological properties of the RbT RBC β -AR using binding/displacement assays. Competitive displacement of ^3H -CGP from RBC β_{3b} -ARs by non-selective β -AR agonists shows that the RbT β_{3b} -AR has a higher affinity

for Nadr than Adr (Figure 3.7), a rank order of potency characteristic of β_1 - and β_3 -ARs (Milligan *et al.*, 1994; Lomasney *et al.*, 1995). Of the selective β -AR agonists, only clenbuterol (β_2 -AR selective) produced significant displacement of ^3H -CGP from RBC β -ARs (Figure 3.7). However, it should be noted that in a separate physiological study clenbuterol blocked the β -AR response in trout RBCs indicating that clenbuterol acts as an antagonist at RbT β_{3b} -ARs and not as an agonist (S.F. Perry, pers. commun.). Use of β -AR subtype selective antagonists to displace ^3H -CGP bound to RBC β_{3b} -ARs indicates β_2 -AR binding characteristics while the high affinity of the RbT β_{3b} -AR for the non-selective β -AR antagonists propranolol and nadolol (Figure 3.8) is not consistent with mammalian β_3 -ARs that show low affinity for these same compounds (Strosberg, 1997). The results of these studies indicate that the binding properties of the trout β_{3b} -AR are “atypical” relative to those of the three pharmacologically defined mammalian β -AR subtypes. Preliminary pharmacological characterization of trout β_{3a} -AR binding in gill suggests the binding properties of β_{3a} -AR are similar to those of β_{3b} -AR (S.G. Dugan, pers. commun.).

Comparisons of trout and mammalian β -ARs at the molecular and pharmacological levels previously showed that the putative trout β_2 -AR is homologous to the mammalian β_2 -AR (Nickerson *et al.*, 2001). Here, we show that the trout β_{3a} - and β_{3b} -ARs are homologous to mammalian β_3 -ARs. At the level of sensitivity to PKA-mediated uncoupling of receptor/G-protein interactions, RbT β_3 -ARs and mammalian β_1 - and β_2 -ARs appear to have similar sensitivities whereas at the level of sensitivity to β ARK-mediated sequestration and down-regulation, RbT β_{3a} -AR resembles mammalian

β_3 -ARs while RbT β_{3b} -AR resembles mammalian β_1 - and β_2 -ARs. At the level of tissue expression patterns, trout β_{3a} -AR exhibits a broad tissue distribution similar to that of mammalian β_1 - and β_2 -ARs while the restricted tissue expression pattern of RbT β_{3b} -AR is similar to the mammalian β_3 -AR. Biochemical characterization of RbT β_{3b} -AR indicates binding properties that are inconsistent with those of any mammalian β -AR subtype.

Our classification of the two RbT β -AR genes described here as β_3 -subtypes is supported by phylogenetic analysis but not by analysis of receptor phosphorylation profiles, tissue specific expression patterns, or binding properties. However, we feel strongly that the classification of these trout β -ARs should reflect their evolutionary relationship to other vertebrate β -ARs. The molecular data reported here indicate the presence of two RbT β_3 -AR genes, which brings the total number of trout β -AR genes to three. The presence of three trout β -ARs with distinct molecular and pharmacological properties supports the presence of a complex and flexible β -AR signaling system in the tissues of this species.

Chapter 4: Cloning of Rainbow Trout β ARK and the Effect of Hypoxia, Cortisol and Clenbuterol on Trout β -AR mRNA Expression Levels

Introduction

β -Adrenoceptor desensitization is a well known and highly characterized phenomenon that occurs following extended periods of β -AR activation and results in a decreased tissue response to catecholamines (Liggett and Raymond, 1993; Freedman *et al.*, 1995; Hein and Kobilka, 1995; Valdenaire and Vanier, 1997). Uncoupling of receptor/G-protein interaction, sequestration of receptors on to internal cellular membranes and down-regulation or reduction of β -AR protein are the three mechanisms that combine to produce β -AR desensitization (Liggett and Raymond, 1993; Lomasney *et al.*, 1995). As previously discussed (Chapter 1, “**Regulation of signaling**”p. 11), each regulatory mechanism occurs over a different time scale and all are triggered by PKA/PKC or β ARK-mediated receptor phosphorylation.

In order to recover catecholamine sensitivity the effects of uncoupling, sequestration and down-regulation must be reversed. Desensitization caused by receptor/G-protein uncoupling and sequestration can be quickly removed by dephosphorylating and recycling β -ARs back to the plasma membrane (Ferguson, 2001; MacDonald and Lefkowitz, 2001). However, restoration of catecholamine sensitivity following β -AR down-regulation requires significantly longer time periods as *de novo* synthesis of β -AR protein is needed (Lomasney *et al.*, 1995; Valdenaire and Vanier, 1997). During this prolonged recovery period organisms may be susceptible to subsequent stressors as the protective or beneficial effects of catecholamine signaling

through β -ARs is compromised. β -AR down-regulation can pose serious problems for organisms that are subject to repeated bouts of acute stress or are chronically stressed. For example, numerous studies of human heart disease reveal that cardiac β_1 -ARs become down-regulated due to the prolonged periods of catecholamine exposure associated with the sub-optimal performance of the failing heart (Bristow *et al.*, 1990; Kompa *et al.*, 1999; White *et al.*, 2000; Port and Bristow, 2001). Down-regulation of cardiac β_1 -ARs results in loss of the ability of catecholamines to restore cardiac performance, leading to the possibility complete cardiac failure (White *et al.*, 2000). Due in large part to its association with heart disease, the mechanism of β -AR down-regulation has been extensively studied in mammalian models.

In the mammalian model of β -AR down-regulation, loss of β -AR protein occurs by increased trafficking of sequestered receptors to lysosomes for proteolytic decay (Tsao and von Zastrow, 2000). Reduced β -AR mRNA half-life represents a second process that can lead to reduced receptor protein levels. Previous studies showed that β -AR mRNA half-life is reduced by binding of destabilizing proteins to the β -AR transcripts (Port *et al.*, 1992; Pende *et al.*, 1996; Tholanikunnel and Malbon, 1997), and β -AR signaling activity has been linked to transcriptional activation of some of these mRNA destabilizing genes (Pende *et al.*, 1996). The final process that may contribute to β -AR down-regulation is a decreased rate of β -AR gene transcription. This process is mediated by PKA-activation of transcription factors that interact with the β -AR gene promoter and exert a negative effect on the rate of transcriptional initiation (Tsao and von Zastrow, 2000; Port and Bristow, 2001). Down-regulation in a particular tissue may be the result of one of these processes acting alone or some combination of all three processes.

Down-regulation of β -ARs in fish was demonstrated in trout red blood cells that showed a reduced number of plasma membrane β -ARs as a result of prolonged exposure to stress (Perry *et al.*, 1996). In addition, our previous molecular analysis of trout β -ARs reveals similarities in the location and frequency of potential PKA/PKC and β ARK phosphorylation sites between trout and mammalian β -ARs (Figures 3.4. and 3.5). Taken together these results suggest that trout β -ARs are subject to down-regulation by the same mechanisms as their mammalian counterparts; this theory receives further support from the cloning of a β -arrestin homolog from trout red blood cells (Jahns *et al.*, 1996). Despite these results the current picture of β -AR down-regulation in trout remains unclear as several questions remain unanswered. For example, although the study by Perry *et al.* (1996) addressed the question of β -AR down-regulation in the broad sense of reduced numbers of plasma membrane β -AR binding sites by using pharmacological methods, these authors did not address the question of which of the three possible down-regulatory processes or combination of processes led to the observed loss of binding sites. Furthermore, although the presence of a trout β -arrestin has been demonstrated in red blood cells, its distribution in other trout tissues remains unknown and no study has shown the presence of a trout β ARK. Finally, differences in the potential phosphorylation profiles between the three trout β -ARs suggests that these receptors may show different sensitivities to down-regulation, a theory that is supported by previous physiology studies using trout cardiac and red blood cell β -ARs (Gamperl *et al.*, 1994; 1998; Perry *et al.*, 1996).

To provide a broader understanding of the mechanisms that contribute to β -AR down-regulation in trout and fish in general, this study examines the presence and tissue

distribution of trout β ARK and β -arrestin and the effect of various treatments on tissue β -AR mRNA levels. Trout β ARK partial cDNA clones were isolated and northern blot experiments were performed to determine the tissue distribution of β ARK and β -arrestin in trout tissues. Experiments to determine the effect of various treatments on trout tissue β -AR mRNA levels are also reported. The presence of β ARK and β -arrestin in most trout tissues examined suggests that at the protein level trout β -ARs are down-regulated by the same mechanisms as mammalian β -ARs, while the results of the mRNA quantitation studies suggest that reduction of β -AR mRNA does not significantly contribute to down-regulation in trout tissues.

Materials and Methods

Animals

Female juvenile rainbow trout (*Oncorhynchus mykiss*), weighing approximately 75-80 g, were obtained from Linwood Acres Trout Farm (Campellcroft, ON). Fish were transported to the University of Ottawa Aquatic Care Facility and were maintained in fiberglass holding tanks (110-115 L) of well aerated, dechloraminated City of Ottawa tap water at 13.0 ± 1.0 °C. Fish were subjected to a constant 12L:12D photoperiod and fed five times weekly with commercial trout pellets.

Experimental Treatments

Environmental Hypoxia. In these experiments, fish were exposed to 24 h of environmental hypoxia ($PwO_2 = 60$ mmHg). Hypoxic conditions were achieved by bubbling nitrogen through a water-gas equilibration column prior to the water entering the box housing the fish. The water volume in the box housing the fish was held at approximately 3 L.

Clenbuterol. Following an acclimation period of approximately one month, fish were separated into two groups of 20-21 fish per 110-115 L tank. Fish were fed twice daily (total of 1.5% BW/day) with either a control or a clenbuterol diet for 37 days. Diets consisted of commercial trout pellets (Martin Mills 5 PT, size 5 mm) treated with carrier only (95% EtOH solution containing the antioxidant ascorbic acid at 0.1 mg/mL) for the control group or with carrier plus clenbuterol (95% EtOH solution containing the antioxidant ascorbic acid at 0.1 mg/mL and 96 mg of clenbuterol/2Kg of pellets) for the treated group. Trout pellets were treated, by careful and even spraying, with 25 mL of carrier solution or carrier solution plus clenbuterol. After 37 days of treatment, fish were

killed with a sharp blow to the head and plasma and liver samples were isolated and stored at -80°C . Plasma samples were assayed for clenbuterol using an EIA kit for clenbuterol (Neogen, Lexington, KY).

Cortisol. Fish were anaesthetized in a 1:12,000 (weight/volume) solution of benzocaine (ethyl-paminobenzoate) cooled to 10°C . After cessation of breathing movements, the fish was transferred to an operating table and the gills were irrigated with the same anesthetic solution throughout the brief period (< 1 min) required to inject cortisol implants. To permit chronic elevation of circulating cortisol levels to a target level of 200-300 ng/mL, fish were injected intraperitoneally with 150 mg/Kg body weight (injection volume 2 mL/Kg) of cortisol (hemisuccinate salt) dissolved in coconut oil (Perry and Reid, 1994). Control fish were injected with equivalent volumes of coconut oil. Control and cortisol-treated fish were kept in separate tanks (100 L) for 4 days prior to blood sampling and tissue removal.

Molecular Cloning

Rainbow trout β ARK partial cDNA clones were amplified from total RNA of RBCs and liver in separate RT-PCR experiments using degenerate primers β ARK5' and β ARK3' (Table 4.1). The β ARK primers were designed from a multiple sequence alignment of mammalian β ARK sequences available from GenBank. Total RNA samples used in the amplification of β ARK and β -arrestin clones were treated with DNase I (DNA free™ kit Ambion, Austin, TX, USA). Synthesis of blood and liver cDNA was done using the 1st Strand cDNA Synthesis Kit for RT-PCR (Roche Molecular Biologicals) with oligo-p(dt)₁₅ primer. PCR amplifications were performed using the

Table 4. 1 Primers used for amplification of rainbow trout BARK, β -arrestin, and β -actin partial cDNA clones.

	5' primers	Pos.	3' primers	Pos.	Ann. Temp. °C
RbTBARK	5'GGGGGTTTGGGGGARGTITAYGGITG ³	597-622 ¹	5'GCRTTIACVTCYCCKICKIGGIG ³	1435-1413 ¹	50
RbTBarr	5'CCIGTIGAYGGIGTIGTIVTIGTIGA ¹	108-133 ²	5'GTVTCIGCISWIGGYTGIGGICCGG ³	559-534 ²	50
RbTB-act	5'ATTAAGGTGACACTATAGAAGVGGGAGC ACGGCATCGTCACC ³	275-292 ¹	5'TAATACGACTCACTATAGGGAGACCGCGGC CGGCCAGATCCAG ³	610-592 ³	50

Sequences are given according to the IUPAC code.

¹ Position of primer relative to the sequence of human BARK2, genbank accession NM_005160.

² Position of primer relative to the complete sequence of trout β -arrestin genbank accession U48410.

³ Position of primer relative to the complete sequence of trout β -actin, genbank accession AJ438158.

following regimen of denaturing, annealing, and extension: 1 x 2 min at 94°C, 30 x (30 sec at 94°C, 30 sec at 50°C, 1 min at 72°C), and 1 x 10 min at 72°C. A total of six trout β ARK clones, three from blood and three from liver, were sequenced on both DNA strands. Trout β -arrestin partial cDNA clones were RT-PCR amplified from total RNA of blood using degenerate primers β -arr5' and β -arr3' (Table 4.1). The trout β -arrestin primers were designed using the complete sequence of the trout β -arrestin isoforms available from GenBank (accession U48410, U48411, and U48412). Three partial trout β -arrestin clones were sequenced on both DNA strands. BLAST searches were performed to confirm the identity of the trout β ARK and β -arrestin clones.

RNA Isolation

Total tissue RNA was isolated using Trizol reagent (Gibco BRL, Burlington, ON, Canada) and RNA quality and quantity were verified by gel electrophoresis and spectrophotometry. RNA used for RPA experiments was treated with the DNA Free™ kit (Ambion).

Northern Blots

Northern blots of trout tissue RNA samples were prepared using the Northern Max™ kit (Ambion, Austin, TX, USA). Fifteen μ g of total RNA from trout gill, heart, kidney, liver, red muscle, white muscle, blood and spleen were loaded on a 14 cm, 1.0% formaldehyde denaturing gel and run at 95 V for 2.5 h in MOPS running buffer. Following electrophoresis RNA was transferred overnight to a positively charged nylon membrane, Bright Star™ (Ambion, Austin, TX, USA). Membranes were prehybridized for approximately 2 h in 10 mL of Ultrahyb™, hybridization solution (Ambion, Austin, TX, USA). Trout β ARK and β -arrestin plasmid clone inserts were gel isolated and used

as templates in probe labeling reactions. Radiolabeled β ARK and β -arrestin probes were synthesized using the Redi-Prime kit (Amersham Pharmacia) and ^{32}P -dCTP. Radiolabeled β ARK and β -arrestin probes were hybridized separately to trout total tissue RNA northern blots in 10 mL of hybridization solution, Ultrahyb™ (Ambion) for 16 h at 42°C. Blots were washed 3 times in 1 L of 2 x SSC, 1.0% SDS at room temperature for 10 min, followed by 3 additional washes in 500 mL of 0.1 x SSC, 0.05% SDS at 65°C for 5 min. Blots were then exposed to autoradiography for 16 h.

RNase Protection Assay

Trout liver, gill, and blood tissue β -AR mRNA levels were measured using the semiquantitative RNase protection assay RPA III™ (Ambion) and gene specific probes for trout β -ARs hybridized in tandem with a trout β -actin probe. The trout β -AR subtype specific probes used in the RPA experiments corresponded to the third intracellular loop region of the three trout β -ARs; amplification of these probes has been described in Chapters 2 (β_2 -AR) and 3 (β_{3a} -, β_{3b} -AR). The trout β -actin probe was amplified from a previously isolated plasmid stock of cloned trout β -actin using primers RbT β act5' and RbT β act3' (Table 4.1). Primers used to amplify the β -actin probe template incorporated the promoter sequences for T7 or SP6 RNA polymerase (Table 4.1) so that sense or antisense RNA could be transcribed. Radiolabeled antisense probes were transcribed using MAXIscript™ (Ambion) with T7 RNA polymerase and ^{32}P -UTP (Amersham Pharmacia). Full-length probes were isolated from a denaturing 4% polyacrylamide, 8 M urea gel. Approximately 7.5×10^4 cpm of β -AR probe and 0.15×10^4 cpm of β -actin probe were hybridized in tandem to 20 μg of total RNA for approximately 16 h at 42°C. Non-hybridized transcripts were digested with approximately 0.4 units of RNase A and

15 units of RNase T1 at 37°C for 90 min. Protected fragments were resolved on a denaturing 6% polyacrylamide, 8 M urea gel that was dried and subjected to autoradiography.

Expression levels for trout tissue β -AR and β -actin were measured as band densities. Following electrophoresis RPA gels were dried and exposed to a phosphor screen (Kodak) for 16-18 h. The screen was then scanned using a phosphor imager, Imager FX (BioRad) and the densities of the β -AR and β -actin bands were measured using the Quantity One software package (version 4.0.3, BioRad). β -AR expression levels for control and treatment samples were expressed as the ratio of β -AR band density versus β -actin band density. The mean ratio of β -AR band density versus β -actin band density was calculated for control and treatment groups and these mean ratios were compared using the Mann-Whitney rank sum test (Sigma Stat, version 2.03, Jandel Scientific).

Results

An 840 bp partial cDNA clone of trout β ARK was RT-PCR amplified from red blood cell and liver RNAs in separate PCR experiments. Sequence comparisons indicate that trout red blood cell and liver β ARK are identical across the 840 base region. The partial trout β ARK clone corresponds to the protein kinase catalytic domain of the human β ARK genes and spans a region that extends from amino acid position 199 to 478 in the human β ARK2 sequence. Within this 279 amino acid region the trout β ARK shares 94% amino acid identity with human β ARK2 and 91% identity with human β ARK1. Northern blot experiments using total RNA from gill, heart, kidney, liver, red muscle, white muscle, blood and spleen indicate that trout β ARK is expressed in all tissues examined with the highest levels of expression in the blood and spleen (Figure 4.1A).

Reverse transcriptase-PCR amplification of a trout β -arrestin fragment produced a band of the expected size in the blood; however, no bands were apparent in amplifications using liver RNA as template (results not shown). The partial trout β -arrestin cDNA clone corresponded to a region spanning amino acids 37 to 187 of the complete coding sequence of the rainbow trout β -arrestin gene (accession U48410, U48411, and U48412). Within this 150 amino acid region the trout β -arrestin sequence shares an 84% and 76% amino acid identity with human β -arrestin2 and β -arrestin1, respectively. Northern blot experiments using the trout partial β -arrestin clone as a probe indicate that trout β -arrestin gene is expressed at relatively high levels in the blood and spleen at lower levels in gill, heart and kidney; there is no detectable expression in liver, red muscle or white muscle (Figure 4.1B).

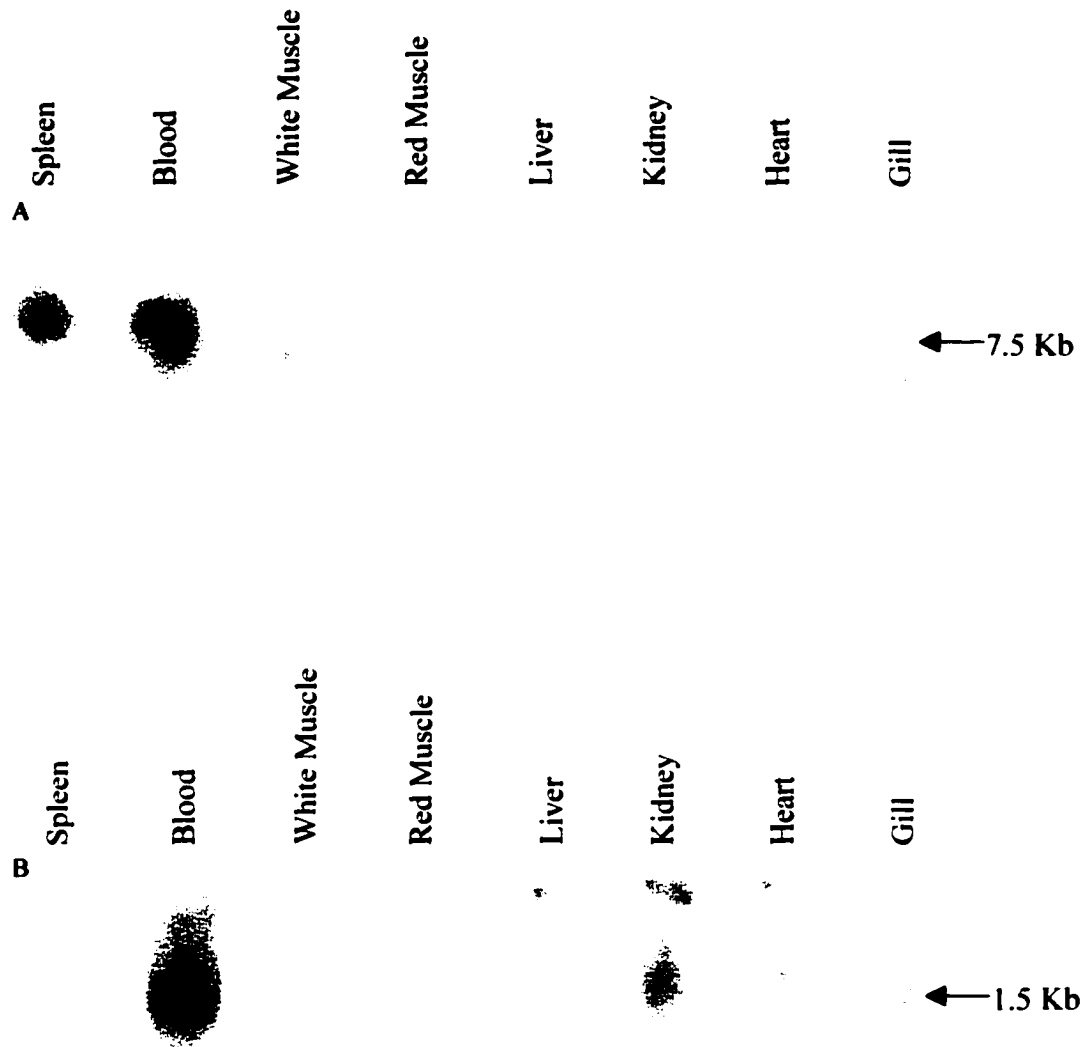


Figure 4.1 (A) Northern blot showing tissue expression pattern of the trout β ARK gene in eight different trout tissues. (B) Northern blot showing the tissue expression pattern of the trout β -arrestin gene in eight different trout tissues.

The effects of hypoxia, clenbuterol, and cortisol treatments on β -AR mRNA expression in trout gill, heart and liver were examined using the RNase protection assay. Hybridization of RbT β_2 -AR, β_{3a} -AR, β_{3b} -AR, and β -actin gene specific probes yielded the expected banding pattern, in most cases. In some RNA samples hybridization of β -AR and β -actin probes produced additional bands. Because the additional β -actin band is not present in all samples within a particular set of hybridizations it is most likely produced by the presence of multiple β -actin alleles within a particular sample. In contrast the additional bands that originate from the β -AR probes tend to be present in all samples within a particular set of hybridizations suggesting the presence of two populations of probe that differ slightly in length but not in sequence. The most likely explanation for the presence of these β -AR probe size variants is premature termination during probe synthesis that results in two β -AR transcripts of similar size that were co-purified during the probe isolation step of the RPA.

For each treatment the mean ratio of β -AR band density/ β -actin band density was calculated in the control and treatment groups. Statistical analysis of these mean ratios indicates no significant difference between control and treatment for hypoxia (hepatic β_2 -AR, Figure 4.2; gill β_{3a} -AR, Figure 4.3; red blood cell β_{3b} -AR, Figure 4.4), clenbuterol (hepatic β_2 -AR, Figure 4.5) or cortisol (hepatic β_2 -AR, Figure 4.6; gill β_{3a} -AR, Figure 4.7; red blood cell β_{3b} -AR, Figure 4.8).

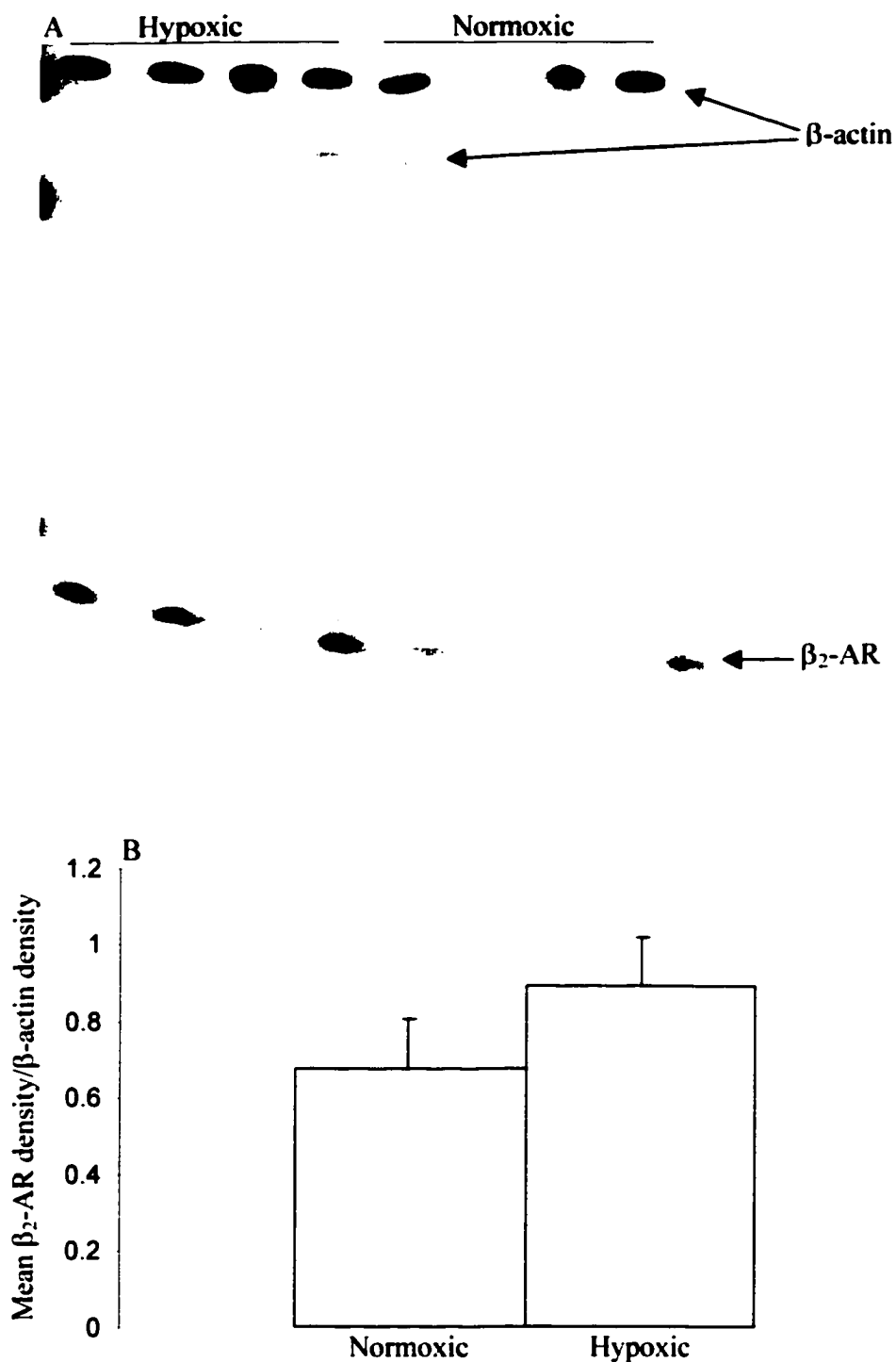


Figure 4.2 (A) RNase protection assay showing the effect of hypoxia on trout hepatic β_2 -AR mRNA relative to β -actin. Each lane represents a sample from a different individual. (B) Mean ratio of β_2 -AR band density/ β -actin band density for normoxic and hypoxic groups. Sample size is three and four for normoxic and hypoxic groups, respectively, and error bars represent standard error. $P=0.629$.

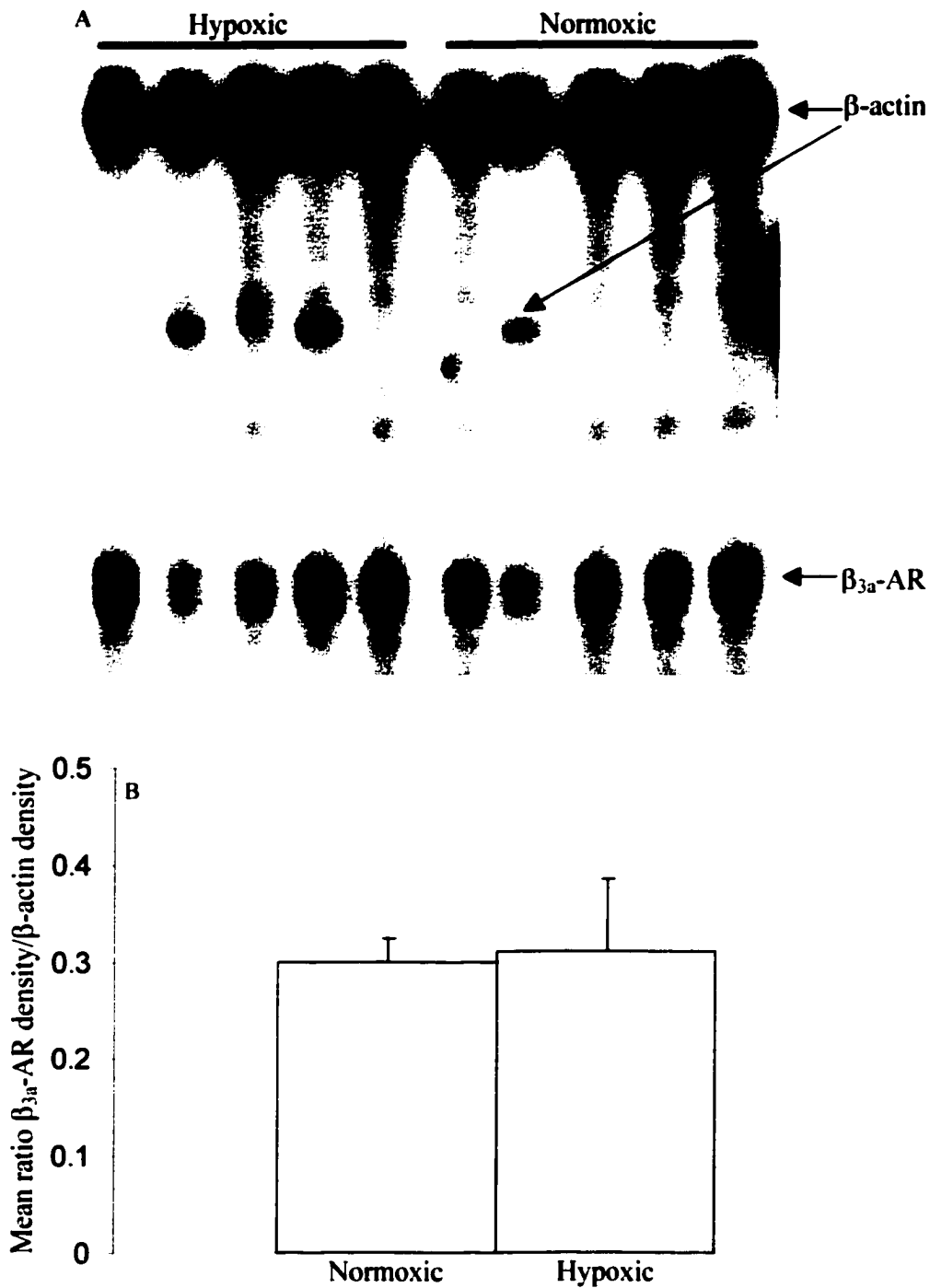


Figure 4.3 (A) RNase protection assay showing the effect of hypoxia on trout gill β_{3a} -AR mRNA relative to β -actin. Each lane represents a sample from a different individual. (B) Mean ratio of β_{3a} -AR band density/ β -actin band density for normoxic and hypoxic groups. Sample size is five for both groups and error bars represent standard error. $P=0.690$.

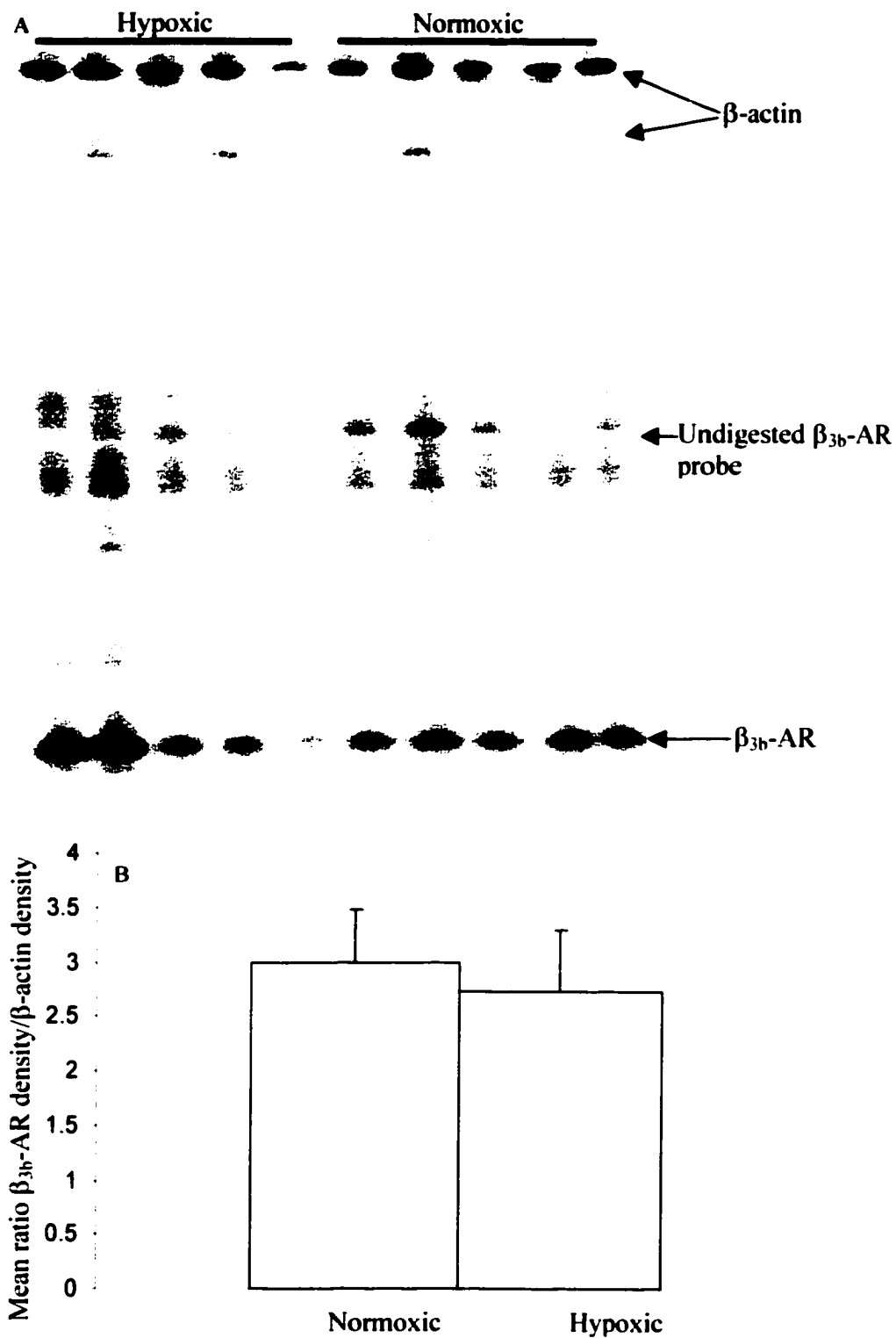


Figure 4.4 (A) RNase protection assay showing the effect of hypoxia on trout red blood cell β_{3b} -AR mRNA relative to β -actin. Each lane represents a sample from a different individual. (B) Mean ratio of β_{3b} -AR band density/ β -actin band density for normoxic and hypoxic groups. Sample size is five for both groups, and error bars represent standard error. $P=0.689$.

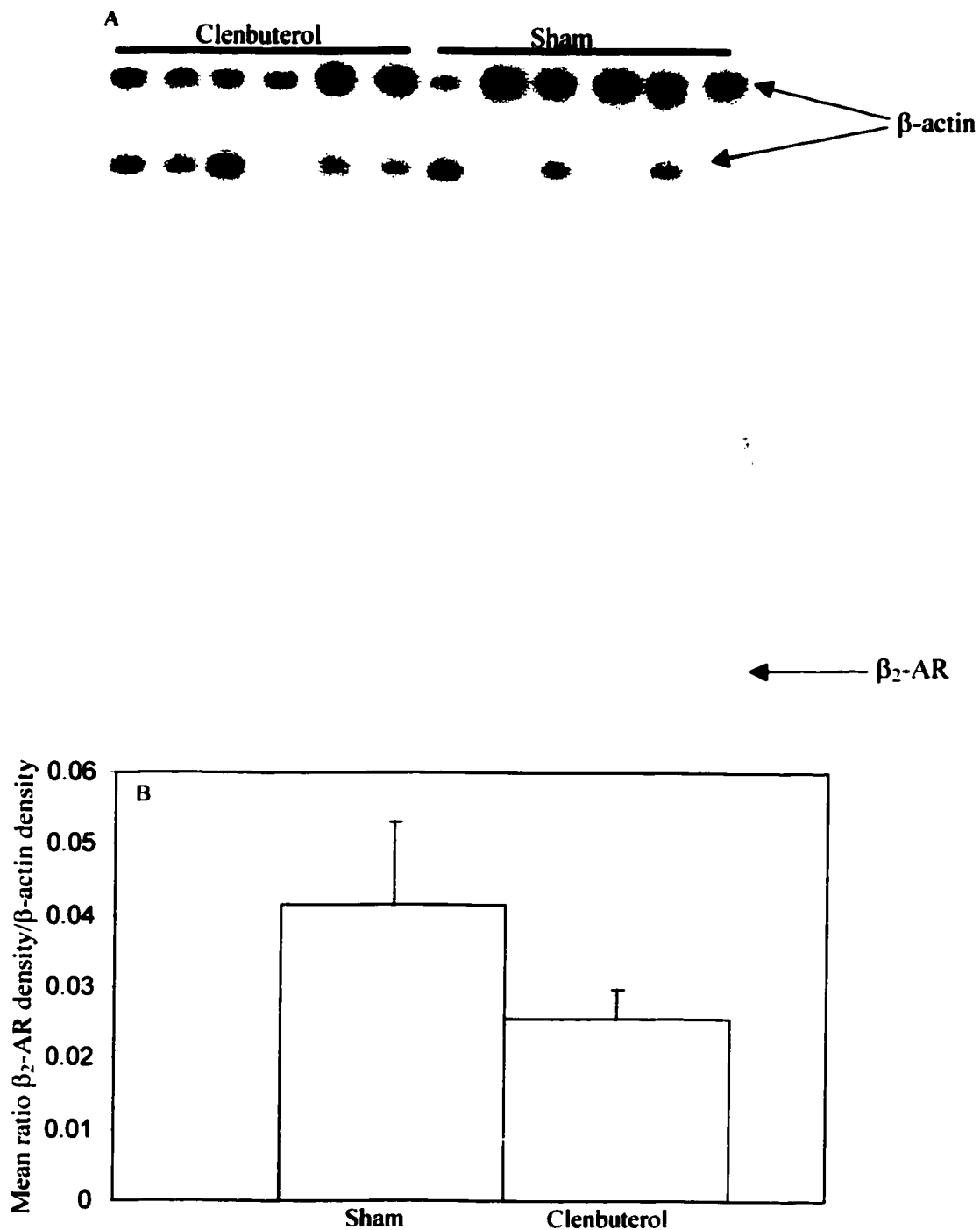


Figure 4.5 (A) RNase protection assay showing the effect of clenbuterol administration on trout hepatic β_2 -AR mRNA relative to β -actin. Each lane represents a sample from a different individual. (B) Mean ratio of β_2 -AR band density/ β -actin band density for control and clenbuterol groups. Sample size is six for both groups, and error bars represent standard error. $P=0.180$.

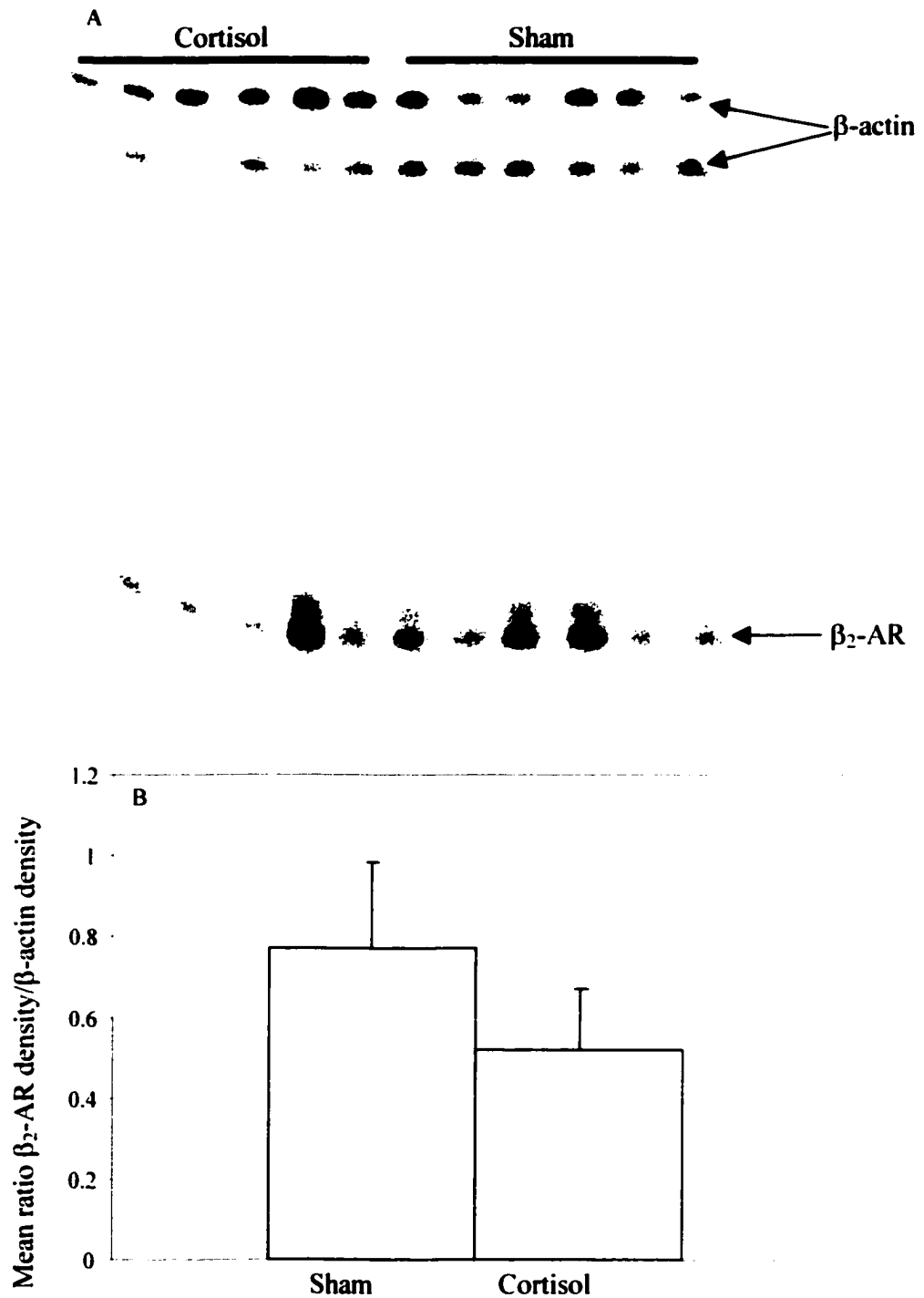


Figure 4.6 (A) RNase protection assay showing the effect of cortisol administration on trout hepatic β_2 -AR mRNA relative to β -actin. Each lane represents a sample from a different individual. (B) Mean ratio of β_2 -AR band density/ β -actin band density for control and cortisol groups. Sample size is six for both groups, and error bars represent standard error. $P=0.180$.

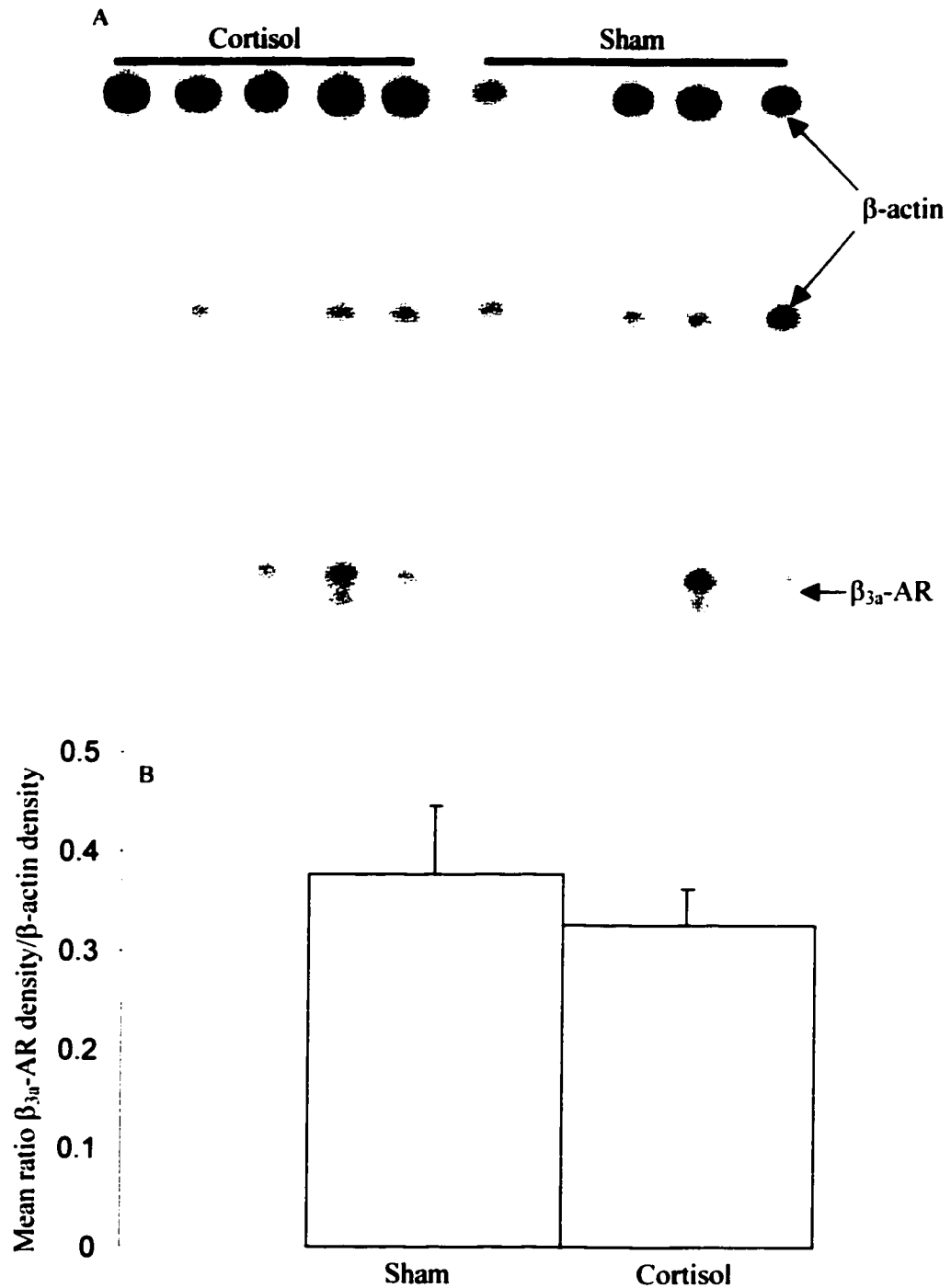


Figure 4.7 (A) RNase protection assay showing the effect of cortisol administration on trout gill $\beta_{3a}\text{-AR}$ mRNA relative to β -actin. Each lane represents a sample from a different individual. (B) Mean ratio of $\beta_{3a}\text{-AR}$ band density/ β -actin band density for sham and cortisol groups. Sample size is four and five for sham and cortisol groups respectively, and error bars represent standard error. $P=0.413$.

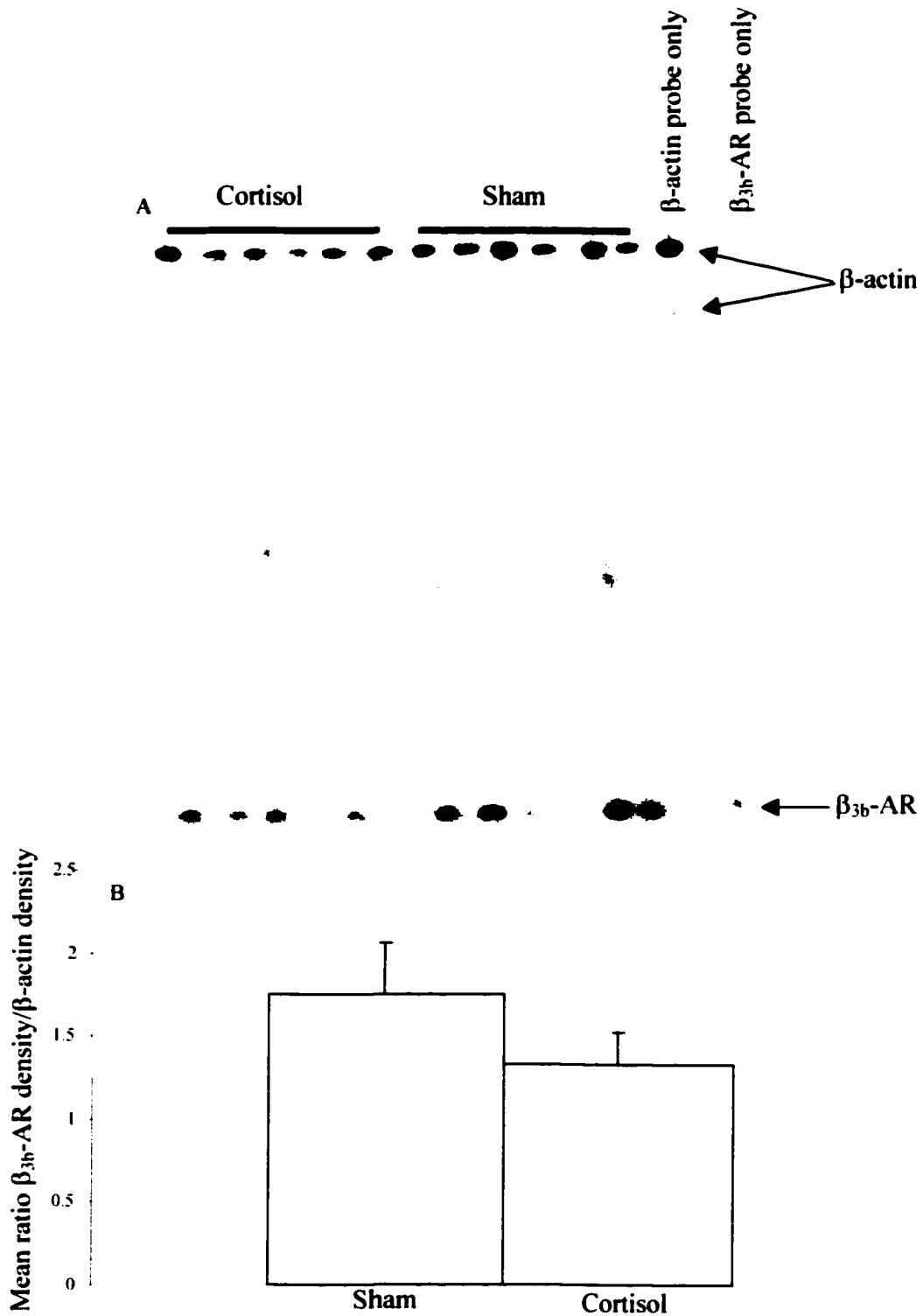


Figure 4.8 (A) RNase protection assay showing the effect of cortisol administration on trout red blood cell β_{3b} -AR mRNA relative to β -actin. Each lane represents a sample from a different individual. (B) Mean ratio of β_{3b} -AR band density/ β -actin band density for sham and cortisol groups. Sample size is five for both groups, and error bars represent standard error. $P=0.310$.

Discussion

With the exception of avian cardiac myocytes (Marsh and Sweeney, 1989) and amphibian and trout red blood cells (Chuang and Costa, 1979; Perry *et al.*, 1996), the question of β -AR down-regulation in non-mammalian vertebrates remains largely unexplored at the molecular and physiological levels. The cloning of a trout β ARK and the distribution of β ARK and β -arrestin in trout tissues shown in this study suggests the existence of a β ARK/ β -arrestin regulatory pathway in this species. In addition, the lack of detectable changes in tissue β -AR mRNA levels following exposure to a prolonged period of hypoxia or following the administration of the β_2 -AR agonist clenbuterol, suggests that reduction of β -AR mRNA levels does not contribute to down-regulation in trout tissues.

In mammals β ARK and β -arrestin are known to play a vital role in the down-regulatory process by providing the trigger for receptor internalization (Ferguson, 2001; MacDonald and Lefkowitz, 2001). In addition to its role in receptor internalization the β ARK/ β -arrestin system has also been implicated in the intracellular targeting of β -ARs to lysosomes. In order to be recycled back to the plasma membrane internalized receptors must first be dephosphorylated by endosomal phosphatases (Lomasney *et al.*, 1995; Mayor *et al.*, 1998; Macdonald and Lefkowitz, 2001). In the model of β ARK/ β -arrestin-mediated targeting of β -ARs to lysosomes the presence of β -arrestin bound to the receptor blocks the access of the phosphatases thus β -ARs remain phosphorylated resulting in their targeting to lysosomes (Macdonald and Lefkowitz, 2001).

The best evidence for the existence of a β ARK/ β -arrestin pathway for receptor internalization in fish comes from the trout red blood cell. Molecular cloning of a trout

β ARK in the present study and the previous cloning of trout β -arrestin (Jahns *et al.*, 1996) demonstrate the presence of these genes in the trout. Northern blot analysis indicates that β ARK and β -arrestin are generally expressed in the same trout tissues and the highest expression level for both β ARK and β -arrestin occurs in the blood (Figure 4.1). Additional support for the existence of the β ARK/ β -arrestin system in the trout comes from previous analysis of trout β -AR amino acid sequences that indicated the presence of a number of potential phosphorylation sites on the cytoplasmic tails of the trout β_2 - and β_{3b} -ARs (Figure 3.5). In a multiple sequence alignment of vertebrate β -AR cytoplasmic tails the position of potential phosphorylation sites in the trout β_2 - and β_{3b} -ARs is similar to known β ARK sites in mammalian β -ARs (Figure 3.5). The trout β_{3b} -AR has the highest number of potential phosphorylation sites in its cytoplasmic tail relative to trout β_2 - and β_{3a} -ARs and it is interesting that RbT β_{3b} -AR is expressed specifically in the blood, the same tissue where the highest levels of β ARK and β -arrestin expression occurs. Furthermore, loss of β -AR binding sites from trout red blood cell plasma membranes following repeated bouts of physical stress was demonstrated by pharmacological studies (Perry *et al.*, 1996). Taken together these results argue strongly for the presence of a β ARK/ β -arrestin pathway of β -AR internalization in the trout red blood cell. It should be noted that a previous study of desensitization in the trout red blood cell did not detect the expected shift in the cellular distribution of β -arrestin from the cytosol to the plasma membrane during desensitization (Jahns *et al.*, 1996). A possible explanation for this discrepancy could be that Jahns *et al.* (1996) assayed red cells after 10 and 30 min of β -AR activation while the loss of red blood cell β -AR binding sites reported by Perry *et al.* (1996) occurred after seven days of repeated stress.

In terms of variable sensitivity to down-regulation between trout β -AR subtypes, a previous analysis of potential receptor phosphorylation profiles suggested that trout β_2 - and β_{3b} -ARs may be subject to β ARK-mediated down-regulation while β_{3a} -AR may be resistant to this same regulatory mechanism (Chapters 2 and 3). In addition to a low abundance of cytoplasmic tail phosphorylation sites, the trout β_{3a} -AR has a truncated cytoplasmic tail relative to trout β_2 - and β_{3b} -ARs. The truncated tail of the mammalian β_3 -AR has been implicated in the resistance of this subtype to down-regulation in mammals (Emorine *et al.*, 1991; Liggett and Raymond, 1993; Strosberg, 1993). The presence of a β -AR in trout cardiac myocytes resistant to down-regulation was suggested from pharmacological studies by Gamperl *et al.* (1994; 1998). It is interesting to note that β_{3a} -AR is expressed highly in the trout heart and furthermore, northern blot analysis of β ARK and β -arrestin expression indicated that these two regulatory proteins are not highly expressed in the trout heart. These results support the theory of a β -AR in the trout heart resistant to down-regulation. On the other hand the β_{3b} -AR has the longest cytoplasmic tail and the highest frequency of potential phosphorylation sites among trout β -ARs. The trout red blood cell expresses a high level of β_{3b} -AR, β ARK and β -arrestin and trout blood is the only tissue where down-regulation of β -AR protein has been demonstrated unequivocally (Perry *et al.*, 1996), thus supporting the theory that β_{3b} -AR is subject to β ARK/ β -arrestin-mediated down-regulation. The sensitivity of trout β_2 -AR to β ARK/ β -arrestin-mediated down-regulation remains in question as northern blot experiments indicate that β -arrestin is not expressed in trout liver. However, a recent study did demonstrate reduced hepatic β_2 -AR binding following treatment with the β_2 -

AR agonist clenbuterol (Dugan and Moon, in preparation). It is suggested that an endocytosis-independent pathway for β -AR protein proteolysis exists in mammalian cells (Jockers *et al.*, 1999). Phosphorylation of the trout β_2 -AR by PKA or other protein kinases at several potential cytoplasmic tail phosphorylation sites (Figure 3.5) may serve as the trigger for an endocytosis-independent pathway of hepatic β_2 -AR down-regulation: the presence of such a pathway awaits further evidence. The contribution of the β ARK/ β -arrestin pathway to β -AR down-regulation in other trout tissues remains in question as β -AR down-regulation remains largely unexplored at the physiological level in most trout tissues.

In some mammalian tissues reduced levels of β -AR mRNA contribute to β -AR down-regulation. Reduction in β -AR mRNA levels result from destabilization of β -AR mRNA and/or reduced rates of β -AR gene transcription (Pende *et al.*, 1996; Tholanikunnel and Malbon, 1997; Tsao and von Zastrow, 2000; Port and Bristow, 2001). To determine the possible contribution of reduced β -AR mRNA levels to β -AR down-regulation in trout tissues, the effect of hypoxia on β -AR mRNA levels in liver, gill, and blood was examined. These three tissues were used based on previous RNase protection assays showing high expression levels for β_2 -, β_{3a} -, and β_{3b} -ARs in trout liver, gill and blood, respectively (Figures 2.3 and 3.6). RNase protection assays using trout β -AR subtype specific probes and β -actin as a control indicated no significant differences in ratios of β -AR mRNA to β -actin levels in liver, gill, or blood of trout subjected to hypoxia versus control fish (Figures 4.2, 4.3, and 4.4, respectively). A second experiment using hepatocytes from trout fed the β_2 -AR agonist clenbuterol produced a

similar finding, although a trend towards a clenbuterol-mediated decrease in hepatic β_2 -AR mRNA was observed (Figure 4.5). This trend is consistent with a pharmacological study of hepatic membranes from these same fish that demonstrate a significant clenbuterol-mediated decrease in the number of hepatic β_2 -AR binding sites on membranes of treated versus control fish (Dugan and Moon, in preparation).

The absence of a detectable stress-induced reduction in tissue β -AR mRNA levels may indicate that reductions in mRNA levels play no role in trout β -AR down-regulation. However, an alternate theory is the possibility that β -AR mRNA levels were reduced in these fish before the study began. Social interactions amongst trout held in captivity cause increased in plasma cortisol levels in some individuals (Sloman *et al.*, 2001). Furthermore, a study of two closely related salmonid species, the rainbow trout and coho salmon, demonstrated a significantly higher number of cardiac β -ARs in the wild coho salmon versus captive rainbow trout (Gamperl *et al.*, 1998). Thus captivity itself may represent a stressor that could cause down-regulation of trout β -ARs at the protein and/or mRNA levels. It seems reasonable to assume that β -AR mRNA levels would fluctuate within a certain range and that during periods of β -AR mRNA down-regulation transcript levels would be near the lower end of this range. If β -AR mRNA levels were already low in the trout used in the above study, then additional stressors such as hypoxia would not produce further reduction in β -AR mRNA levels. If this were the case treatments known to positively effect trout tissue β -AR numbers should produce detectable increases in β -AR mRNA levels. To explore this possibility cortisol was administered to trout as previous studies reported this treatment increased the total cellular content of β -AR protein in red blood cells and liver (Reid and Perry, 1991; Reid *et al.*, 1992). However,

this study found no significant change in β -AR mRNA levels in liver, gill or blood of cortisol treated versus control trout (Figures 4.6, 4.7, and 4.8, respectively) suggesting that increased transcription is not necessary for an increase in protein.

The presence of a trout β ARK and the similarities in the tissue distributions of β ARK and β -arrestin in trout tissues reported in this study provides some support for the hypothesis that trout β -ARs are subject to the same regulatory mechanisms as their mammalian counterparts at the level of receptor protein. The molecular requirements for β ARK/ β -arrestin-mediated β -AR down-regulation are most prevalent in the trout red blood cell, a tissue where β -AR down-regulation has been previously demonstrated at the pharmacological level (Perry *et al.*, 1996). Characterization of trout tissue β -AR mRNA levels following various treatments indicates that modulation of β -AR protein levels in trout tissues does not appear to occur at the transcript level. However the presence of additional regulatory mechanisms seems likely.

Chapter 5: General Summary

Extensive characterization of mammalian β -ARs at the levels of pharmacology and molecular biology over the past 50 years has provided a comprehensive understanding of the complexity and flexibility of the mammalian β -AR system. These studies demonstrated the presence of three distinct mammalian β -AR subtypes (β_1 -, β_2 - and β_3 -ARs) and revealed that in general β_1 -ARs mediate positive chronotropic and inotropic responses of the heart (Rohrer, 1998), β_2 -ARs control glucose release from the liver (García-Sàinz, *et al.*, 1996), and β_3 -ARs mediate the thermogenic response of brown and white adipose tissue (Strosberg *et al.*, 1997).

The work presented here shows that the rainbow trout also possesses three distinct β -ARs but that these are different from those of mammals. In contrast to mammals the trout appears not to possess a β_1 -AR subtype, but does possess one β_2 -AR and two β_3 -ARs (β_{3a} - and β_{3b} -AR). The presence of multiple pharmacologically distinct β -ARs in trout tissues was suggested by previous studies (Reid *et al.*, 1992; Gamperl *et al.*, 1994; Gilmour *et al.*, 1994), however, the cloning of three distinct trout β -AR genes in the present study provides the first conclusive evidence for three β -AR genes in trout. The absence of a β_1 -AR in fish is supported not only by the fact that we were unable to amplify a trout β_1 -AR gene but also by the absence of this gene in the available sequence of the puffer fish genome. Interestingly, the expression of trout β_{3a} -AR in several tissues including heart suggests that this gene has assumed the function of the missing β_1 -AR gene in trout.

In mammals a physiologically significant role for β_3 -AR signaling is restricted to regulating the thermogenic response of adipose tissues, thus the β_3 -AR subtype is often referred to as being adipose tissue specific, this despite its expression at low levels in other mammalian tissues (Strosberg, 1997). The “atypical” regulatory characteristics of the mammalian β_3 -AR were thought to represent adaptations of this receptor for its specific role in regulating the thermogenic response of adipose tissue. This study is the first to demonstrate the presence of a β_3 -AR homolog in fish, a fact that is of particular interest given that fish have no thermogenic response and that trout was found to possess two β_3 -ARs (β_{3a} - and β_{3b} -AR).

The tissue specific expression patterns of the three trout β -ARs in this study conflict with some previous pharmacological classifications of trout tissue β -ARs. The trout red blood cell β -AR has been previously characterized as a β_1 -AR subtype based on pharmacology (Tetens *et al.*, 1988; Perry and McDonald, 1993); however, the present study clearly demonstrated that the trout red blood cell specifically expresses the β_{3b} -AR subtype. Pharmacological characterization of β_{3b} -AR indicates that this trout receptor has “atypical” binding characteristics relative to those of the three mammalian β -AR subtypes. The high expression level of β_{3a} -AR versus β_2 -AR in the trout heart is also noteworthy as previous studies indicated that signaling in the trout heart was controlled exclusively by a β_2 -AR (Gamperl *et al.*, 1994; Olsson *et al.*, 2000). The expression of a trout β_2 -AR in hepatocytes provides molecular confirmation for the previous pharmacological classification of this receptor as a β_2 -AR subtype.

In terms of regulation of β -AR signaling, previous studies reported that treatments resulting in the prolonged activity of β -ARs cause a reduction in the number of cell surface β -AR binding sites in some trout tissues but not in others (Gamperl *et al.*, 1994; 1998; Perry *et al.*, 1996). These previous pharmacology based studies proposed that the same “classic” mechanisms of mammalian β -AR regulation were also operating in trout tissues. In the mammalian model of β -AR regulation, PKA/PKC and β ARK-mediated receptor phosphorylation and subsequent binding of β -arrestin to the receptor are key components that trigger activation of regulatory mechanisms (Strosberg, 1993; Lomansey *et al.*, 1995). The present study demonstrated the presence of a trout β ARK gene and that trout and mammalian β -AR phosphorylation profiles are similar, thus providing molecular evidence for the presence of “classic” β -AR regulatory mechanisms in some trout tissues. Furthermore, this study provides the first strong evidence for a β ARK/ β -arrestin-mediated pathway of β -AR down-regulation in the trout red blood cell. This finding conflicts with a previous molecular study proposing that the β ARK/ β -arrestin pathway did not contribute to β -AR down-regulation in the trout red blood cell (Jahns *et al.*, 1996). In their study, Jahns *et al.* (1996) were unable to detect a shift in the cellular distribution of β -arrestin from the cytoplasm to the plasma membrane of trout RBCs after 30 minutes of β -AR stimulation and they proposed an alternate role for β -arrestin involving down-regulation of the RBC β NHE. However, a period of 30 minutes may be insufficient to cause β -AR down-regulation in the trout RBC, as loss of RBC β -AR binding sites was demonstrated after seven days of repeated stress (Perry *et al.*, 1996). It seems likely that a β ARK/ β -arrestin pathway of β -AR down-regulation would contribute

to this previously observed reduction of trout RBC β -AR binding sites. In addition, the expression pattern, the phosphorylation profile and the truncated cytoplasmic tail of the trout β_{3a} -AR shown in the present study provides a molecular explanation for the previously observed resistance to down-regulation of the trout cardiac β -AR (Gamperl *et al.*, 1994; 1998).

This molecular characterization of trout β -ARs reveals that the rainbow trout β -AR gene-family is composed of three genes that encode three distinct β -AR subtypes. The three trout β -ARs are classified as β_2 -, β_{3a} - and β_{3b} -ARs based on their evolutionary relationships to mammalian β -ARs in the traditional β_1 -, β_2 -, or β_3 -AR classification. Hybridization of gene specific probes to tissue RNA indicates distinct tissue specific expression patterns for each trout β -AR subtype. Trout β_2 -AR was expressed in all tissues examined with the exception of blood, with the highest levels of RbT β_2 -AR expression seen in the kidney, liver, and red and white muscles (Figure 2.3). Trout β_{3a} -AR is highly expressed in the gill and heart and at lower levels in the kidney and red and white muscles, while RbT β_{3b} -AR is specifically expressed in the blood (Figure 3.6). Pharmacological characterization of trout β_2 - and β_{3b} -ARs indicated that trout β_2 -AR has binding characteristics similar to those of mammalian β_2 -ARs (Figure 2.4) while the binding characteristics of trout β_{3b} -AR are “atypical” with respect to the well-defined pharmacology of mammalian β -AR subtypes (Figure 3.7 and 3.8). The presence and location of potential regulatory phosphorylation sites on trout β -AR subtypes and the expression of a trout β ARK and β -arrestin in tissues where β -AR down-regulation is demonstrated at the pharmacological level, suggests that trout β -AR signaling is

regulated by mechanisms similar to those of mammalian β -AR signaling. However, different potential phosphorylation profiles among trout β -AR subtypes suggest that each subtype may exhibit different susceptibilities to the regulatory mechanisms of receptor/G-protein uncoupling, sequestration, and down-regulation. Modulation of cellular β -AR mRNA levels does not appear to contribute significantly to down-regulation of RbT β_2 -, β_{3a} -, or β_{3b} -ARs in liver, gill, or blood. This study has answered several important questions concerning the molecular biology of the trout β -AR gene-family and provides the first glimpse at the molecular foundation underlying the β -AR system in trout as well as fish in general. The presence of multiple trout β -AR subtypes with specific tissue expression patterns, distinct pharmacologies and different potentials for signal regulation confirms the complexity of the trout β -AR system.

This study provides the first crucial steps towards a more complete understanding of the molecular biology underlying the β -AR system of trout. In addition, several interesting questions are raised that will guide future research in the field of fish β -AR systems. One of the most important issues that need to be addressed is the ability of mammalian β -AR subtype selective compounds to differentiate between different trout/fish β -AR subtypes. The ability to selectively activate or block a particular β -AR subtype will lead to significant advances in our understanding of fish β -AR systems particularly at the *in vivo* level. With respect to the evolution of β -ARs in vertebrates this study raises additional questions. Characterization of the β -AR gene-families from additional species, both teleost and non-teleost, will provide information to confirm the proposed loss of the β_1 -AR gene in the teleost lineage and will also enable us to

determine at what point the β_1 -AR was lost during teleost evolution. These studies may also shed light on the evolution of the β -AR gene-family of vertebrates in general by determining the timing of gene-duplications events that gave rise to the β_1 -, β_2 - and β_3 -AR genes. Finally, by characterizing fish β -ARs we broaden our understanding of one component of the adrenergic system; however, studies to characterize other components of the adrenergic system including α_1 - and α_2 -ARs, G-proteins, and regulatory proteins are also required if we are to advance our understanding of this large and complex system.

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Appendix 3.1 Multiple sequence alignment of selected vertebrate β -AR amino acid sequences including RbT β_2 -, β_{3a} -, and β_{3b} -ARs. Sequences were aligned using Clustal W version 1.8 (Thompson *et al.*, 1994) with default settings. This alignment was used to generate the maximum likelihood tree shown in Figure 3.1 on page 58.

cat β_2 MGQ----PGRSVFLLAPNG-----SHAPDQDGTQ---ERNDAWV-- 33
 dog β_2 MGQ----PANRSVFLAPNG-----SHAPDQDGSQ---ERSEAWV-- 33
 bovine β_2 MGQ----PGRSVFLLAPNA-----SHAPDQDQVTL---ERDEAWV-- 33
 human β_2 MGQ----PGNGSAFLLAPNR-----SHAPDHDVDTQ---ERDEAWV-- 33
 monkey β_2 MGQ----PGNGSAFLLAPNG-----SHAPDHDVDTQ---ERDEAWV-- 33
 mouse β_2 MGP----HGNDSDFLAPNG-----SRAPHDVDTQ---ERDEAWV-- 33
 rat β_2 MEP----HGNDSDFLAPNG-----SRAPHDVDTQ---ERDEAWV-- 33
 pig β_2 MGQ----PGRSVFLLAPNG-----SHAPDQDVPQ---ERDEAWV-- 33
 trout β_2 MEN----VSTPAVFNLSDSL-----VENSSSRQWSEYSEYSEAVA-- 36
 puffer β_2 MGS----TAPTGYLNASTEK-----IPTSDPTKQE---EGYSEVTH-- 34
 cat β_1 MGAGALALGASEPCNLSSAAPLPDGAATAARLLVPASPSPASPLTPTSEGPAPLSQQWT-- 58
 dog β_1 MGAGALALGASEPCNLSSAAPLPDGAATAARLLVPASPSPASPLTPTSEGPAPLSQQWT-- 58
 bovine β_1 MGAGVLALGASEPCNLSSAAPVPDGAATAARLLVPASPSPASPLTPTSEGPAPLSQQWT-- 58
 pig β_1 MGAGALALGASEPCNLSSAAPLPDGAATAARLLVPASPSPASPLTPTSEGPAPLSQQWT-- 58
 mouse β_1 MGAGALALGASEPCNLSSAAPLPDGAATAARLLVLPASPSPASPLTPTSEGPAPLSQQWT-- 58
 rat β_1 MGAGALALGASEPCNLSSAAPLPDGAATAARLLVLPASPSPASPLTPTSEGPAPLSQQWT-- 58
 human β_1 MGAGVLVGLASEPCNLSSAAPLPDGAATAARLLVPASPSPASPLTPTSEGPAPLSQQWT-- 58
 monkey β_1 MGAGALVGLASEPCNLSSAAPLPDGVATAARLLVPASPSPASPLTPTSEGPAPLSQQWT-- 58
 frog β_1 MGDQMGPHPCR---NRSG-----TPTTVSPMHPPLPELTHQWT-- 35
 turkey β_1 MGDGNLPPDCGP-HNRSGG-----GATAAPTSRQVSAKLLSQQWE-- 41
 bovine β_1 MAP--WPPGNSSLTPWPD-----IPTLAPNTANASGLPGVPWA-- 36
 pig β_1 MAP--WPPGNSSLTPWPD-----IPTLAPNTANASGLPGVPWA-- 36
 cat β_1 MAP--WPHGNGSLASWPA-----APTLPNTANTSGLPGVFPA-- 36
 dog β_1 MAP--WPHGNGSVASWPA-----APTLPNTANTSGLPGVFPA-- 36
 human β_1 MAP--WPHENSLLAPWPD-----LPTLAPNTANTSGLPGVFPE-- 36
 monkey β_1 MAP--WPHGNSLVPWPD-----VPTLAPNTANTSGLPGVFPA-- 36
 mouse β_1 MAP--WPHRNGSLALWSD-----APTLDPSAANTSGLPGVFPA-- 36
 rat β_1 MAP--WPHRNGSLAFWSD-----APTLDPSAANTSGLPGVFPA-- 36
 turkey β_{1c} MTP--LPAGNGSVFNCSSW-----AAVLSRQWA----- 25
 trout β_{1a} MHEKSILPLCMSFLMKMDFNLSSIV-----LYAVNTVTVPFHNTGPYHPAAG 48
 trout β_{1b} MHEKSILPLCMSFLMKMDFNLSSIV-----LYAVNTVTVPFHNTGPYHPAAG 48
 puffer β_1 MMDNHLN-----DTNRYARYY-AAGPWS----- 22
 amphioxus d/ β MSANTTVSPTEETTANLTAN-----STEASVGSCFAPNPYSAGV-- 38
 *
 cat β_2 ---VG-MGIVMSLIVLAIVFGNVLVITAIARPERLQTVTNYFITSLACADLVMGGLAVVVF 89
 dog β_2 ---VG-MGIVMSLIVLAIVFGNVLVITAIARPERLQTVTNYFITSLACADLVMGGLAVVVF 89
 bovine β_2 ---VG-MGILMSLIVLAIVFGNVLVITAIARPERLQTVTNYFITSLACADLVMGGLAVVVF 89
 human β_2 ---VG-MGIVMSLIVLAIVFGNVLVITAIARPERLQTVTNYFITSLACADLVMGGLAVVVF 89
 monkey β_2 ---VG-MGIVMSLIVLAIVFGNVLVITAIARPERLQTVTNYFITSLACADLVMGGLAVVVF 89
 mouse β_2 ---VG-MAILMSVIVLAIVFGNVLVITAIARPERLQTVTNYFITSLACADLVMGGLAVVVF 89
 rat β_2 ---VG-MAILMSVIVLAIVFGNVLVITAIARPERLQTVTNYFITSLACADLVMGGLAVVVF 89
 pig β_2 ---VG-MAIVMSLIVLAIVFGNVLVITAIARPERLQTVTNYFITSLACADLVMGGLAVVVF 89
 trout β_2 ---VL-LGILMALLVMKIVFGNVLVITAIARPERLQTVTNYFITSLACADLVMGGLAVVVF 92
 puffer β_2 ---IL-LGMVMSFLVLCIVFGNVLVITAIARPERLQTVTNYFITSLACADLVMGGLAVVVF 90
 cat β_1 ---AG-IGLLMALIVLLIVAGNVLVIVAIARTPRLOTLTNLFIMSLASADLVMGGLVVPF 114
 dog β_1 ---AG-IGLLMALIVLLIVAGNVLVIVAIARTPRLOTLTNLFIMSLASADLVMGGLVVPF 114
 bovine β_1 ---AG-MGLLMAFIVLLIVAGNVLVIVAIARTPRLOTLTNLFIMSLASADLVMGGLVVPF 114
 pig β_1 ---AG-MGLLMAFIVLLIVAGNVLVIVAIARTPRLOTLTNLFIMSLASADLVMGGLVVPF 114
 mouse β_1 ---AG-MGLLVALIVLLIVAGNVLVIVAIARTPRLOTLTNLFIMSLASADLVMGGLVVPF 114
 rat β_1 ---AG-MGLLVALIVLLIVAGNVLVIVAIARTPRLOTLTNLFIMSLASADLVMGGLVVPF 114
 human β_1 ---AG-MGLLMAFIVLLIVAGNVLVIVAIARTPRLOTLTNLFIMSLASADLVMGGLVVPF 114
 monkey β_1 ---AG-MGLLMAFIVLLIVAGNVLVIVAIARTPRLOTLTNLFIMSLASADLVMGGLVVPF 114
 frog β_1 ---MG-MTHFMAAIIILLIVMGNMIVVAIGRNQRLQTLTNVFITSLACADLVMGFLVVP 91
 turkey β_1 ---AG-MSLLMALVLLIVAGNVLVIVAIARTPRLOTLTNLFIMSLASADLVMGGLVVPF 97
 bovine β_1 ---VALAGALLALAVLATVGGNLLVIVAIARTPRLOTLTNVFTSLATADLVVGLLVVP 93
 pig β_1 ---VALAGALLALAVLATVGGNLLVIVAIARTPRLOTLTNVFTSLATADLVVGLLVVP 93
 cat β_1 ---VALAGALLALAVLATVGGNLLVIVAIARTPRLOTLTNVFTSLATADLVVGLLVVP 93
 dog β_1 ---VALAGALLALAVLATVGGNLLVIVAIARTPRLOTLTNVFTSLATADLVVGLLVVP 93
 human β_1 ---AALAGALLALAVLATVGGNLLVIVAIARTPRLOTLTNVFTSLAAADLVVGLLVVP 93
 monkey β_1 ---AALAGALLALAVLATVGGNLLVIVAIARTPRLOTLTNVFTSLAAADLVVGLLVVP 93
 mouse β_1 ---AALAGALLA---LATVGGNLLVIVAIARTPRLOTLTNVFTSLAAADLVVGLLVMP 90
 rat β_1 ---AALAGALLA---LATVGGNLLVIVAIARTPRLOTLTNVFTSLATADLVVGLLVMP 90
 turkey β_{1c} -----VGAALSITILVIVAGNLLVIVAIARTPRLOTLTNVFTSLACADLVMGGLVVP 79
 trout β_{1a} PWSLL----LLVIIIMTIVGNLLVIVAIARTSQLOTLTNIFIMSLGICADLVVGLVVP 104
 trout β_{1b} PWSLL----LVVIIIMTIVGNLLVIVAIARTSQLOTLTNIFIMSLACADLVVGLVVP 104
 puffer β_1 -LV-----IMTIIILVIVGNLLVIVAVTRTSQLOTLTNIFIMSLGICADLVVGLVVP 75
 amphioxus d/ β ---QAVLGLITVILILLTVIGNVLVIVAVTRTSQLOTLTNIFIVSLACADLVVGLVVP 95
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cat β ₂	GASHILHGMWTFGNFWCFWTSIDVLCVTASIELTLCVIAVDRYFAITSPPFKYQSLLTQNK	149
dog β ₂	GASHILHGMWTFGNFWCFWTSIDVLCVTASIELTLCVIAVDRYFAITSPPFKYQSLLTQNK	149
bovine β ₂	GACHILHGMWTFGNFWCFWTSIDVLCVTASIELTLCVIAVDRYLAITSPPFKYQCLLTQNK	149
human β ₂	GAAILHGMWTFGNFWCFWTSIDVLCVTASIELTLCVIAVDRYFAITSPPFKYQSLLTQNK	149
monkey β ₂	GAAILHGMWTFGNFWCFWTSIDVLCVTASIELTLCVIAVDRYFAITSPPFKYQSLLTQNK	149
mouse β ₂	GASHTSHGMWTFGNFWCFWTSIDVLCVTASIELTLCVIAVDRYVAITSPPFKYQSLLTQNK	149
rat β ₂	GASHILHGMWTFGNFWCFWTSIDVLCVTASIELTLCVIAVDRYVAITSPPFKYQSLLTQNK	149
pig β ₂	GASHILHGMWTFGSGFWCFWTSIDVLCVTASIELTLCVIAVDRYLAITSPPFKYQCLLTQNK	149
trout β ₂	GACYILLNTWHPGSLFCEFWTAADVLCVTASIELTLCVIALDRYLAITSPLRYPSSLTKRK	152
puffer β ₂	GACYIIFKTHWFGSFWCFWTAADVLCVTASIELTLCVIAIDRYLAITSPPFRYQSLTKCR	150
cat β ₁	GATIVMGRMEYGSFFCELMTSDVLCVTASIELTLCVIALDRYLAITSPPFRYQSLLTRAR	174
dog β ₁	GATIVMGRMEYGSFFCELMTSDVLCVTASIELTLCVIALDRYLAITSPPFRYQSLLTRAR	174
bovine β ₁	GATIVVDRMEYGSFFCELMTSDVLCVTASIELTLCVIALDRYLAITSPPFRYQSLLTRAR	174
pig β ₁	GATIVVGRMEYGSFFCELMTSDVLCVTASIELTLCVIALDRYLAITSPPFRYQSLLTRAR	174
mouse β ₁	GATIVVGRMEYGSFFCELMTSDVLCVTASIELTLCVIALDRYLAITSPPFRYQSLLTRAR	174
rat β ₁	GATIVVGRMEYGSFFCELMTSDVLCVTASIELTLCVIALDRYLAITSPPFRYQSLLTRAR	174
human β ₁	GATIVVGRMEYGSFFCELMTSDVLCVTASIELTLCVIALDRYLAITSPPFRYQSLLTRAR	174
monkey β ₁	GATIVVGRMEYGSFFCELMTSDVLCVTASIELTLCVIALDRYLAITSPPFRYQSLLTRAR	174
frog β ₁	GATLVVGRMLYGSIFCEFWTSIDVLCVTASIELTLCVIAIDRYLAITSPPFRYQSLTKGR	151
turkey β ₁	GATLVVGRMLYGSIFCEFWTSIDVLCVTASIELTLCVIAIDRYLAITSPPFRYQSLTKGR	151
bovine β ₁	GATLALTGHWPLGVTGCELMTSDVLCVTASIELTLCALAVDRYLAVTNPLRYGALVTKRR	153
pig β ₁	GATLALTGHWPLGVTGCELMTSDVLCVTASIELTLCALAVDRYLAVTNPLRYGALVTKRR	153
cat β ₁	GATLALTGHWPLGATGCELMTSDVLCVTASIELTLCALAVDRYLAVTNPLRYGALVTKRR	153
dog β ₁	GATLALTGHWPLGATGCELMTSDVLCVTASIELTLCALAVDRYLAVTNPLRYGALVTKRR	153
human β ₁	AATLALTGHWPLGATGCELMTSDVLCVTASIELTLCALAVDRYLAVTNPLRYGALVTKRR	153
monkey β ₁	AATLALTGHWPLGATGCELMTSDVLCVTASIELTLCALAVDRYLAVTNPLRYGALVTKRR	153
mouse β ₁	GATLALTGHWPLGETGCELMTSDVLCVTASIELTLCALAVDRYLAVTNPLRYGLVTKRR	150
rat β ₁	GATLALTGHWPLGATGCELMTSDVLCVTASIELTLCALAVDRYLAVTNPLRYGLVTKRR	150
turkey β _{1c}	GATILLSGHMYPYGTVCCELMTSLDVLCVTASIELTLCALAVDRYLAVTNPLRYGALVTKGR	139
trout β _{1a}	GATIVLTGNWQLSDTSCCELMTSDVLCVTASIELTLCVIAVDRYLAITRPLRHKVLLNKR	164
trout β _{1b}	GATIVVTGNWQLSDTSCCELMTSDVLCVTASIELTLCVIAVDRYLAITRPLRHKVLLNKR	164
puffer β ₁	GATIVVTGNWQLSDTSCCELMTSDVLCVTASIELTLCVIAVDRYLAITRPLRHKVLLNKR	135
amphioxus d/β	AATNDILGYWPPGG-YCDVWVSFDVNLNSTASILNLVWIAFDRLAITSPPFYHTRTERT	154
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cat β ₂	ARVVILMHWIVSGLTSFLPIQHWYRAT-HQEAINCYAKETCCDFFTNQAYAIASSIVSF	208
dog β ₂	ARVVILMHWIVSGLTSFLPIQHWYRAT-HQEAINCYAKETCCDFFTNQAYAIASSIVSF	208
bovine β ₂	ARVVILMHWIVSGLTSFLPIQHWYRAS-HQEAINCYAKETCCDFFTNQAYAIASSIVSF	208
human β ₂	ARVILMHWIVSGLTSFLPIQHWYRAT-HQEAINCYANETCCDFFTNQAYAIASSIVSF	208
monkey β ₂	ARVILMHWIVSGLTSFLPIQHWYRAT-HQEAINCYAKETCCDFFTNQAYAIASSIVSF	208
mouse β ₂	ARVVILMHWIVSGLTSFLPIQHWYRAT-HKKAIDCYTEETCCDFFTNQAYAIASSIVSF	208
rat β ₂	ARVVILMHWIVSGLTSFLPIQHWYRAT-HKQAIDCYAKETCCDFFTNQAYAIASSIVSF	208
pig β ₂	ARVVILMHWVVSGLISFLPIQHWYQAT-HREALNCYAEACDFFTNQAYAIASSIVSF	208
trout β ₂	ACVVVVTVWVAALISFLPIHGMWVSD-EPEALSCLEDAHCCDFFTNAYAVASSVVSF	211
puffer β ₂	ARIVVVLWVVAIALISFLPIHGMWISD-DEEAKRCIEDSSCFEYTNMAYAITSSIIISF	209
cat β ₁	ARALVCTVWAIASALVSFLPILMHWRAE-GDEARRCYNDPKCCDFVTNRAYAIASSVVSF	233
dog β ₁	ARALVCTVWAIASALVSFLPILMHWRAE-GDEARRCYNDPKCCDFVTNRAYAIASSVVSF	233
bovine β ₁	ARALVCTVWAIASALVSFLPIHGMWRDK-DAKAGGCYNDPECCDFINEGYAITSSVVSF	233
pig β ₁	ARALVCTVWAIASALVSFLPILMHWWRDK-GAEARRCYNDPKCCDFVTNRAYAIASSVVSF	232
mouse β ₁	ARALVCTVWAIASALVSFLPILMHWRAE-SDEARRCYNDPKCCDFVTNRAYAIASSVVSF	233
rat β ₁	ARALVCTVWAIASALVSFLPILMHWRAE-SDEARRCYNDPKCCDFVTNRAYAIASSVVSF	233
human β ₁	ARGLVCTVWAIASALVSFLPILMHWRAE-SDEARRCYNDPKCCDFVTNRAYAIASSVVSF	233
monkey β ₁	ARGLVCTVWAIASALVSFLPILMHWRAE-SDEARRCYNDPKCCDFVTNRAYAIASSVVSF	233
frog β ₁	AKGIVCSVNGISALVSFLPIMHWWRDGTGDLAMKCYEDPGCCDFVTNRAYAIASSIIISF	211
turkey β ₁	AKVIICTVWAIASALVSFLPIMHWWRDE-DPQALKCYQDPGCCDFVTNRAYAIASSIIISF	216
bovine β ₁	ALAAVVLVWVSAAVSFAPIMSKMWRVGADAEARQCHSNPRCCTFASNMYPYALLSSSVSF	213
pig β ₁	ARAAVVLVWVSAAVSFAPIMSKMWRVGADAEARQCHSNPRCCTFASNMYPYALLSSSVSF	213
cat β ₁	ARAAVVLVWVSAAVSFAPIMSKMWRVGADAEARQCHSNPHCCAFASNI PYALLSSSVSF	213
dog β ₁	ARAAVVLVWVSAAVSFAPIMSKMWRVGADAEARQCHSNPHCCAFASNI PYALLSSSVSF	213
human β ₁	ARTAVVLVWVSAAVSFAPIMSQMWRVGADAEARQCHSNPRCCAFASNMYPYVLLSSSVSF	213
monkey β ₁	ARAAVVLVWVSAAVSFAPIMSQMWRVGADAEARQCHSNPRCCAFASNMYPYVLLSSSVSF	213
mouse β ₁	ARAAVVLVWVSAAVSFAPIMSQMWRVGADAEARQCHSNPRCCSFASNMYPYVLLSSSVSF	210
rat β ₁	ARAAVVLVWVSAVTSFAPIMSQMWRVGADAEARQCHSNPRCCSFASNMYPYVLLSSSVSF	210
turkey β _{1c}	AWAVVCHVWAIASIFLPIHGMWRDGADEQAVRCYDPRCCDFVTNRAYAIIVSSTVSF	199
trout β _{1a}	ARLIVCVVWVVSALISFVPIHGMWRRAEDDPEAVLCYQDPACCDFLINRTFAISSVVSF	224
trout β _{1b}	ARLIVCVVWVVSALISFVPIHGMWRRAEDDPEAVLCYQDPACCDFLINRTFAISSVVSF	224
puffer β ₁	ARVIVCLVWVSSLSIFVPIHGMWRD-DDRTAKECYADPACCDFTNRAYAIIVSIVSF	194
amphioxus d/β	AGILVATVWVVSALISFVPIHGMWRDQSEALAIYSDPCLCIFTASTAYTIVSLSISF	214
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cat β_2	DN-LIPKE-VYILLNWVGYVNSAFNPLIYCRSPDFRIAFQELLCLRR-----SSLKAYGNG	353
dog β_2	DN-LIPKE-VYILLNWVGYVNSAFNPLIYCRSPDFRIAFQELLCLRR-----SSLKAYGNG	353
bovine β_2	DN-LIRKE-IYILLNWLGYNVNSAFNPLIYCRSPDFRIAFQELLCLRR-----SSLKAYGNG	353
human β_2	DN-LIRKE-VYILLNWIGYVNSGFNPLIYCRSPDFRIAFQELLCLRR-----SSLKAYGNG	353
monkey β_2	DN-LIPKE-VYILLNWVGYVNSGFNPLIYCRSPDFRIAFQELLCLRR-----SSLKAYGNG	353
mouse β_2	DN-LIPKE-VYILLNWLGYNVNSAFNPLIYCRSPDFRIAFQELLCLRR-----SSFETYGNG	353
rat β_2	AN-LIPKE-VYILLNWLGYNVNSAFNPLIYCRSPDFRIAFQELLCLRR-----SSKTYGNG	353
pig β_2	DN-LIPKE-VYILLNWVGYVNSAFNPLIYCRSPDFRIAFQELLCLRR-----SSLKAYGNG	353
trout β_2	KV-DNIRK-PFRILNWIGYANSAFNPLIYCRSPEFRYAFQELLCLRG-----AAPPTNGYI	361
puffer β_2	KE-QHNDL-AFRILNWIGYANSAFNPLIYCRSPEFRYAFKELLCHRNRLTNPFGVNGYI	358
cat β_1	RD-LVPDR-LFVFFNWLGYANSAFNPIIYCRSPDFRKAQORLLCFARRAARGHAAAGDR	404
dog β_1	RD-LVPDR-LFVFFNWLGYANSAFNPIIYCRSPDFRKAQORLLCCARRAARGHGAAGDP	405
bovine β_1	RD-LVPDR-LFVFFNWLGYANSAFNPIIYCRSPDFRKAQORLLCCARRAARGHGAAGDP	395
pig β_1	RD-LVPDR-LFVFFNWLGYANSAFNPIIYCRSPDFRKAQORLLCCARRAARGHGAAGDP	398
mouse β_1	RD-LVPDR-LFVFFNWLGYANSAFNPIIYCRSPDFRKAQORLLCCARRAARGHGAAGDP	397
rat β_1	RD-LVPDR-LFVFFNWLGYANSAFNPIIYCRSPDFRKAQORLLCCARRAARGHGAAGDP	397
human β_1	RE-LVPDR-LFVFFNWLGYANSAFNPIIYCRSPDFRKAQORLLCCARRAARGHGAAGDP	408
monkey β_1	RE-LVPDR-LFVFFNWLGYANSAFNPIIYCRSPDFRKAQORLLCCARRAARGHGAAGDP	411
frog β_1	RN-LIPDK-LFLFLNWLGYNVNSAFNPIIYCRSPDFRKAQORLLCCARRAARGHGAAGDP	353
turkey β_1	RD-LVPDR-LFVFFNWLGYANSAFNPIIYCRSPDFRKAQORLLCCARRAARGHGAAGDP	371
bovine β_1	GP-SLVSGPTFLALNWLGYNVNSAFNPLIYCRSPDFRSAPRLLCRCR---PEEHLAAASP	374
pig β_1	GP-SLVSGPTFLALNWLGYNVNSAFNPLIYCRSPDFRSAPRLLCRCR---PEEHLAAASP	374
cat β_1	GPSLVSP-AFLALNWLGYNVNSAFNPLIYCRSPDFRSAPRLLCRCR---LEERHAAASP	374
dog β_1	GPSLVSP-ALLALNWLGYNVNSAFNPLIYCRSPDFRSAPRLLCRCR---REEHRAASP	374
human β_1	GPSLVSP-AFLALNWLGYNVNSAFNPLIYCRSPDFRSAPRLLCRCR---PEEHLAAASP	377
monkey β_1	GPSLVSP-AFLALNWLGYNVNSAFNPLIYCRSPDFRSAPRLLCRCR---PEEHLAAASP	377
mouse β_1	GPSLVSP-AFLALNWLGYNVNSAFNPLIYCRSPDFRSAPRLLCRCR---PEEHLAAASP	377
rat β_1	GPSLVSP-AFLALNWLGYNVNSAFNPLIYCRSPDFRSAPRLLCRCR---PEEHLAAASP	377
turkey β_{1c}	RP-LVPDQ-LFLFLNWLGYNVNSAFNPIIYCRSPDFRSAPRLLCRCR---PEEHLAAASP	345
trout β_{1a}	RN-VPPGE-IFRLLNWLGYNVNSAFNPIIYCRSPEFRYAFKELLGCPWFSTPRNFIF--YK	396
trout β_{1b}	RN-VPPGE-IFRLLNWLGYNVNSAFNPIIYCRSPEFRYAFKELLGCPWFSTPRNFIF--YK	396
puffer β_1	RE-VPSK-IFRLLNWLGYNVNSAFNPIIYCRSPEFRYAFKELLGCPWFSTPRNFIF--YK	363
amphioxus d/ β	DR-CVQPA-VFIALTWLWINSFNPPIIYAFNKEFRKVFVKMICCHKRGVTVGPNHADL	350
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cat β_2	YSNNSNSRTDYAGEHSGGFLGQEKD-----SEVLCEDPP-----GT	389
dog β_2	YSNNSNSRSDYAGEHSGGFLGQEKD-----SELLCEDPP-----GT	389
bovine β_2	CSSNSNDRDYYTGEQSGYHLGEEKD-----SELLCEDPP-----GT	389
human β_2	YSSN-----GNTGEQSGYHLGEEKD-----NKLLEDLP-----GT	384
monkey β_2	YSSNSN-----GNTGEQSGYHLGEEKD-----NKLLEDLP-----GT	386
mouse β_2	YSSNSNGRTDYTGEQNTCQLGQERE-----QELLCEDPP-----GM	389
rat β_2	YSSNSNGRTDYTGEQSAQQLGQEKD-----NELLCEEAP-----GM	389
pig β_2	CSSNSNGRTDYTGEQSGCYLGEEKD-----SENLCEDAP-----GP	389
trout β_2	YRHSRLRSPKDRPGSLSNVGTVE-----LGLSSVTNINGYCNPPPLASIV----	409
puffer β_2	YSRHSQSEQGRSKVSLDESE-----ANESCLG-----KGM	390
cat β_1	PRASGCLPGTRPPSPGAASDDDDDD-----VGAAPPARL-----LE	442
dog β_1	PRA-----RPPSPGAASDDDDDDDDDDAGAGAGAAPPARL-----LE	442
bovine β_1	PRALGCLAVARPPSPGAASDDDDDD-----EDDVGAPPVRL-----LE	436
pig β_1	PRASGCLAVARPPSPGAASDDDD-----EEDVGAAPPAPL-----LE	437
mouse β_1	PRASGCLARAGPPSPGAPSDDDDD-----AGTTPPARL-----LE	434
rat β_1	PRASGCLARAGPPSPGAPSDDDDD-----AGATPPARL-----LE	434
human β_1	PRASGCLARPGPPSPGAASDDDDDDV-----VGATPPARL-----LE	446
monkey β_1	PRASGCLARPGPPSPGAASDDDDDDV-----VGATQPARL-----LE	449
frog β_1	-----ELSRYSGGFVN-----SLDT---NA-----LG	372
turkey β_1	-----QPAPLPGGFIS-----TLGSPHSP-----GG	393
bovine β_1	-----PRAPSGAPTA-----LTSPAGPMQ-----PP	395
pig β_1	-----PRAPSGAPT-----LTSPAGPRQ-----PS	395
cat β_1	-----AGETSDAPAA-----LTPAESG-----392	
dog β_1	-----PGDPSAAPAA-----LTSPAESSR-----CP	395
human β_1	-----ALFPGVPA-----RSSPAQPR-----CQ	398
monkey β_1	-----ASSPRAPLRF-----GPAPRSPGF-----AS	398
mouse β_1	-----ASPVEARQSP-----PLNRFDGYE-----GA	395
rat β_1	-----ASPVASRONS-----PLNRFDGYE-----GE	395
turkey β_{1c}	QHCS-----CAFSRPGDME-----DSKAVDPGH-----LR	371
trout β_{1a}	ELRTRCPCFLGSAEAG-AASFQKPP-----AAATPEG-----427	
trout β_{1b}	EKRTHCPCFLGSAEAG-AASFQKPP-----AAARPEGDE-----SLA	431
puffer β_1	ELQTRCSCFLQARSSAPGSFHKSP-----ASKSEAS-----LT	397
amphioxus d/ β	N-----YDPVAMRLK-----RGENANGTVN-----GD	373

cat β_2	ENLANRQGTVPN---DSIDSQGGSTNDSLL-----	418
dog β_2	E---DRQGTVPS---DSVDSQGRNCSTNDSLL-----	415
bovine β_2	ENFVNQGGTVPS---DSIDSQGRNCSTNDSLL-----	418
human β_2	EDFVGHQGTVPS---DNIDSQGRNCSTNDSLL-----	413
monkey β_2	EDFVGHQGTVPS---DNIDSQGRNCSTNDSLL-----	415
mouse β_2	EGFVNCQGTVPS---LSVDSQGRNCSTNDSPL-----	418
rat β_2	EGFVNCQGTVPS---LSIDSQGRNCSTNDSPL-----	418
pig β_2	EGCAHRQGTVPD---DSTDSQGRNCSTNDSPL-----	418
trout β_2	-----	
puffer β_2	EVCTVIDRKTVDP-----NGNCRKGLTTVTTL-----	417
cat β_1	FWAGCNGGAAAADSDSSLDPEGRPGASESKV-----	474
dog β_1	FWAGCNGG-AAADSDSSLDGAGSPAGASESKV-----	473
bovine β_1	FWAGYNGG-AAANSDDSPDEPSRAGASESKV-----	467
pig β_1	FWAGYNGG-AAARDSDDLDEPTPGGRASESKV-----	468
mouse β_1	FWGCGNGGTTTVDSDSSLDPEGRQGFSSSESKV-----	466
rat β_1	FWAGCNGGTTTVDSDSSLDPEGRQGFSSSESKV-----	466
human β_1	FWAGCNGG-AAADSDSSLDPECRPGASESKV-----	477
monkey β_1	FWAGCNGG-AAADSDSSLDPECRPGASESKV-----	480
frog β_1	MCSECNG-----VRTSLD-----	385
turkey β_1	TWSDCNGG-TRGGSESSLEERHSKTSRSESIEMERKNILATTTFYCTFLGNGDKAVFCTV	452
bovine β_3	KLDGASCGLS-----	405
pig β_3	PLDGLPADFL-----	405
cat β_3	LPGGIS-----	398
dog β_3	ALDGVN-----	401
human β_3	RLDG-----	402
monkey β_3	GSTGLLGEFLRPEGQBATLR-----	418
mouse β_3	RPFPT-----	400
rat β_3	RPFPT-----	400
turkey β_{ec}	EDSEVQSGRRREENASSHGGGHQORPLGECWLQGNQSHLCEQLDEPTSTENPAGPSV----	428
trout β_{3a}	-----	
trout β_{3b}	SSQSSDKSEEPSGPPPHNGNTQFSEFSKPENELNWTGEKLTFTTD-----	477
puffer β_3	SNQESNKGEVSPGPRSNGSTPEFDLSEQ-----	427
amphioxus d/ β	ANGKANGNIEAGEGTSSS-----	391

cat β_2	-----
dog β_2	-----
bovine β_2	-----
human β_2	-----
monkey β_2	-----
mouse β_2	-----
rat β_2	-----
pig β_2	-----
trout β_2	-----
puffer β_2	-----
cat β_1	-----
dog β_1	-----
bovine β_1	-----
pig β_1	-----
mouse β_1	-----
rat β_1	-----
human β_1	-----
monkey β_1	-----
frog β_1	-----
turkey β_1	LRIVKLFEDATCTCPHTHKLKQWRPKQHQ 483
bovine β_3	-----
pig β_3	-----
cat β_3	-----
dog β_3	-----
human β_3	-----
monkey β_3	-----
mouse β_3	-----
rat β_3	-----
turkey β_{ec}	-----
trout β_{3a}	-----
trout β_{3b}	-----
puffer β_3	-----
amphioxus d/ β	-----