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THE REGULATION OF β-ADRENOCEPTORS IN TWO TELEOST FISHES

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ABSTRACT

Exposure of an organism to a stressor results in a primary stress response. A part of this response involves the release of the catecholamines (CA), adrenaline (ADR) and noradrenaline (NADR), which exert their actions by binding to specific membrane binding sites or receptors termed adrenergic receptors or adrenoceptors (ARs). Continuous exposure to a hormone results in a decrease in the responsiveness of the cell. This process, termed desensitization may reduce the cell's responsiveness only to that hormone (homologous) or to another agonist (heterologous). Moreover, prolonged agonist exposure may result in the internalization (sequestration) and possible degradation of the receptor (down-regulation). These processes have been well studied for the three β-AR subtypes in mammals, however little is known about how β-ARs are regulated in teleost fish.

The goal of this thesis was to examine the regulation of the β-ARs in two species of teleosts, the rainbow trout (*Oncorhynchus mykiss*) and the black bullhead (*Ameiurus melas*). Fish were exposed to various stressors including prolonged agonist exposure, physical exertion and hypercarbia and receptor binding characteristics [affinity (K_d) and number of binding sites (B_{max})] as well as function were examined. Chasing to exhaustion and prolonged feeding of the β-agonist clenbuterol significantly reduced the B_{max} of hepatic β_2-ARs in rainbow trout by 27% and 33%, respectively. In contrast, exposure to hypercarbia did not significantly affect rainbow trout gill β-ARs. Pharmacological characterization of the gill β-AR supports an atypical classification compared with the three classic mammalian subtypes. No significant differences were observed in binding parameters for bullhead hepatic β-ARs after a 24 hour exposure to the β-agonist isoproterenol or a 1 hour air-exposure. However, air-exposure did result in a moderate desensitization in CA-stimulated

hepatic glucose production. Phylogenetic analysis groups the bullhead hepatic β-AR with the mammalian β_3-ARs, which is not supported by the pharmacological classification of a β_2-AR subtype. This study was the first to comparatively examine β-AR regulation for different subtypes of teleost β-ARs. Findings suggest β-ARs in teleosts possess different sensitivities to down-regulation and desensitization as is seen with the different mammalian β-AR subtypes.

RÉSUMÉ

Suite à un stress, les organismes réagissent à l'aide de réponse physiologique à un stress. Une partie cette réponse consiste à une augmentation des niveaux sanguins d'hormones de stress appelées catécholamines (CAs), soit l'adrénaline (ADR) et la noradrénaline (NADR), pour ensuite agir directement sur des récepteurs membranaires spécifiques nommés récepteurs adrénergiques ou adrénocepteurs (ARs). Un niveau élevé d'hormone pour une période donnée peut diminuer la réponse cellulaire à cette hormone. Ce processus résulte en une atténuation de la réponse cellulaire ou désensibilisation a cette même hormone (homologue) ou une atténuation de la réponse cellulaire a d'autres agonistes (hétérologue). De plus, suite à un stress prolongé il peut se produire une internalisation (séquestration) qui peut être suivis d'une dégradation des récepteurs (régulation négative). Ce mécanisme a été fortement étudié pour trois types de β-ARs chez les mammifères, toutefois très peu d'information existe à ce sujet chez les téléostéens.

Cette thèse a pour but d'examiner la régulation de β-ARs chez deux espèces téléostéens, la truite arc-en-ciel (*Oncorhynchus mykiss*) et la barbotte noir (*Ameiurus melas*). Les poissons on été exposés à différents stress tels des traitements prolongés d'agonistes adrénergiques, un effort physique soutenu et des conditions d'hypercarbia et l'affinité (K_d) et le nombres de sites de fixations (B_{max}) ainsi que la fonctionnalité des ARs furent déterminés. L'exercice jusqu'a épuisement ainsi que les traitements prolongés de l'agoniste adrénergique clenbuterol ont diminué significativement le B_{max} des β_2-ARs hépatiques chez la truite arc-en-ciel de 27% et 33%, respectivement. En revanche, des conditions d'hypercarbia n'ont pas affecté les β-ARs des branchies chez la truite arc-en-ciel. Les caractéristiques pharmacologiques des β-ARs des branchies chez la truite arc-en-ciel démontrent une

classification atypique comparativement à la classification en trois sous-types de β-ARs connus chez les mammifères. Aucune différences au niveau des paramètres de fixation des β-ARs hépatiques ne furent observées chez la barbotte noir suite à des traitements à l'agoniste isoproterenol (24 h) ainsi qu'à des périodes d'exposition à l'air (1 h). Par contre, suite à des périodes d'exposition à l'air une désensibilisation modérée de la production de glucose hépatique par stimulation de CA fut observée chez la barbotte noir. Une analyse phylogénétique des β-ARs hépatiques chez la barbotte noir indiquent une similarité prononcée avec les β_3-ARs de mammifères, ce qui est différent des résultats obtenus suite à la caractérisation pharmacologique; indiquant des propriétés similaires aux β_2-ARs de mammifères. Cette étude est la première à examiner de façon comparative la régulation de différent sous-type de β-ARs de téléostéens. Les observations suggèrent que les β-ARs téléostéens possède différentes sensibilités à la régulation négative et la désensibilisation observées chez les mammifères.

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LIST OF ABBREVIATIONS

ACase	adenylyl cyclase
ADR	adrenaline
ALP	alprenolol
ANOVA	analysis of variance
AR	adrenoceptor
ATL	atenolol
ATP	adenosine triphosphate
AUAP	abridged universal amplification primer
BAAM	bromoacetyl alprenolol menthane
βARK	β-adrenoceptor kinase
BCA	bicinchoninic acid
BSA	bovine serum albumin
CA	catecholamine
cAMP	cyclic adenosine monophosphate
CLEN	clenbuterol
CLO	clonodine
COMT	catechol-*o*-methyl transferase
CTL	control
d	day
DAG	1,2-diacylglycerol
DBH	dopamine-β-hydroxylase
DHA	dihydroalprenolol
DOB	dobutamine
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol- bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EIA	enzyme immunoassay
FBS	fetal bovine serum
FSK	forskolin
GDP	guanosine diphosphate
GPase	glycogen phoshorylase

GPCR	G-protein coupled receptor
GRK	G-protein receptor kinase
GTP	guanosine triphosphate
GTPase	guanosinetriphosphatase
h	hour
IP_3	inositol 1,4,5-trisphosphate
ISO	isoproterenol
MAO	monoamine oxidase
min	minute
MR	mitochondria-rich
mRNA	messenger ribonucleic acid
NADR	noradrenaline
PCR	polymerase chain reaction
PHE	phenylephrine
PHT	phentolamine
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PNMT	phenylethanolamine-N-methyl transferase
PROC	procaterol
PROP	propanolol
PRZ	prazosin
PVC	pavement cell
RACT	ractopamine
RBC	red blood cell
RIA	radioimmunoassay
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SEM	standard error of the mean
YOH	yohimbine

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Chapter 1: General Introduction

1.1 Rationale of study

In teleosts, previous research on adrenergic receptors or adrenoceptors (ARs) has focused on their characterization and function in various tissues but in a limited number of species. Inhabiting an aquatic environment, fish are often exposed to a wide variety of stressors. Catecholamines (CAs) and ARs are an integral component in the stress response and play a vital role in ameliorating the effect of stressors to restore normal body processes (Randall and Perry, 1992; Fabbri and Moon, 1994; Wendelaar Bonga, 1997). Yet, little is known concerning the regulation of the ARs and how different stressors affect AR properties in teleosts.

In mammals, the various β-AR subtypes show different sensitivities to desensitization and down-regulation (Marullo *et al.*, 1995). In particular, the β_2-AR displays a relatively high degree of desensitization and is often the model receptor used in G-protein coupled receptor (GPCR) regulation studies (Lohse, 1993). Research into the phenomena of desensitization and down-regulation has concentrated primarily on mammalian β-ARs but very little is known concerning the regulation of this process in non-mammalian vertebrates, including teleosts.

Therefore, the purpose of this research was directed towards understanding the regulation of the β-ARs in two representative teleosts species, the rainbow trout (*Oncorhynchus mykiss*) and the black bullhead (*Ameiurus melas*). These two species have very different natural histories and have been the species of choice in previous AR studies. In addition, a significant species difference in the responsiveness of the β-ARs was

previously demonstrated (Fabbri *et al.*, 1995a, b). Thus, the objective of this study was to test the hypotheses that

1. differences exist in the regulation of a single β-AR subtype between the rainbow trout and the black bullhead; and,
2. subtype differences exist in the regulation of β-ARs in the rainbow trout.

1.2 The adrenergic system

ARs are members of the large superfamily of plasma membrane bound heptahelical receptors that are coupled to a family of guanine nucleotide regulatory proteins (G-proteins). Members of this superfamily include a wide variety of receptors for many peptide hormones, some neurotransmitters and sensory stimuli (Strosberg, 1993; Birnbaumer and Birnbaumer, 1995). Of the five classes of GPCRs, ARs belong to the aminergic family. ARs are characterized by consisting of a single polypeptide chain of 400 to over 500 amino acids, possessing seven hydrophobic membrane spanning domains with each domain consisting of 20 to 25 amino acids. The seven membrane spanning domains are connected by three intra- and three extracellular loops, an extracellular N-terminal head and an intracellular C-terminal tail (Strosberg, 1993; Barnes, 1995). The first and second extracellular loops are connected by a disulfide bridge that is thought to play an important role in the conformation of the receptor (Wallukat, 2002). The lengths of the hydrophilic loops, head and tail are subtype specific and as well show the most divergence in amino acid sequence (Strader *et al.*, 1989). A representative AR structure is shown in Figure 1.1.

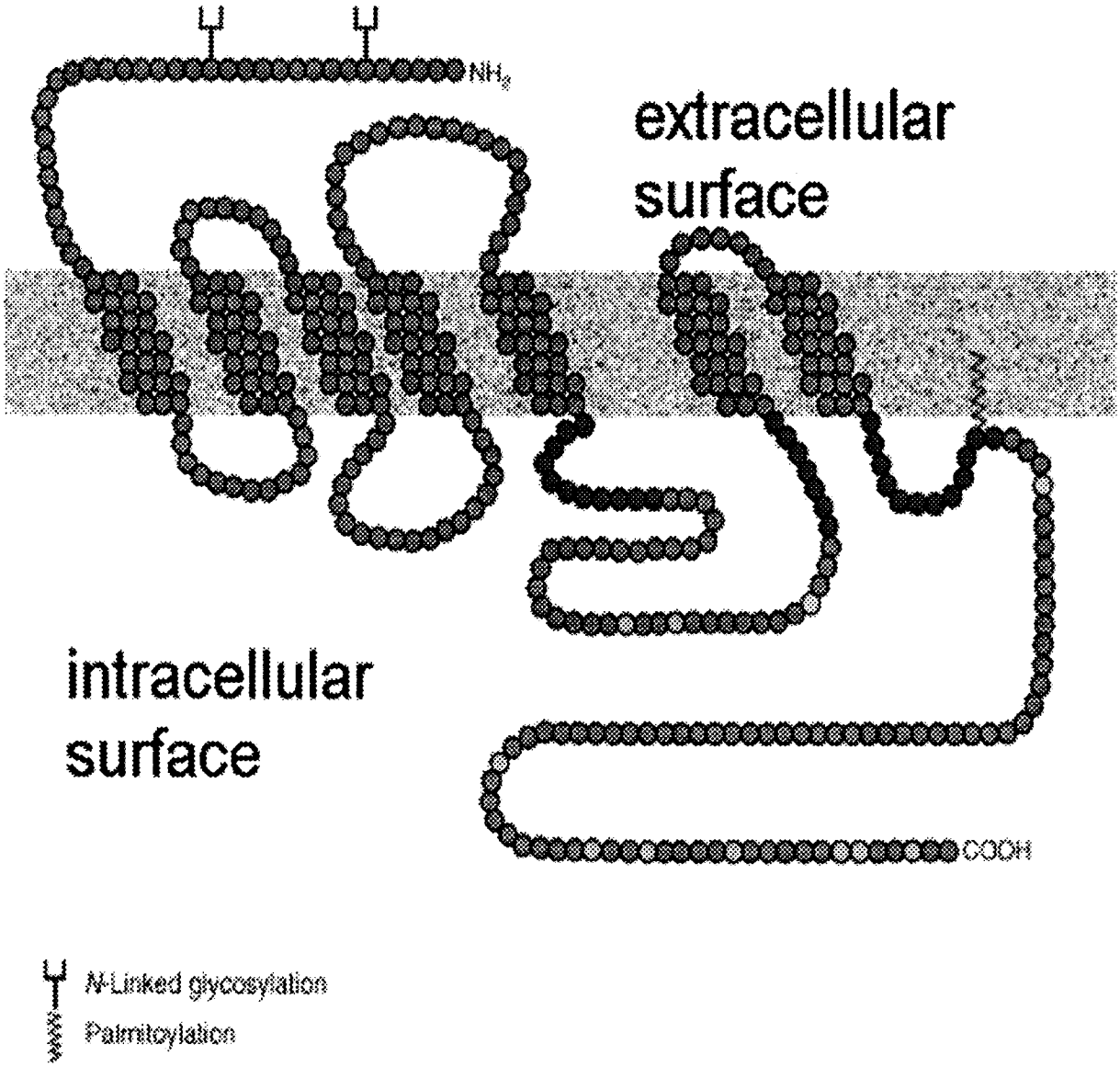


Figure 1.1. Structure of a mammalian β_2-AR showing the seven transmembrane spanning domains, extracellular amino head and intracellular carboxy tail. Figure is modified from Lefkowitz (2000).

Alquist (1948) originally classified these receptors pharmacologically into just two types, α and β, based on their relative affinities for adrenaline (ADR), noradrenaline (NADR), and isoproterenol (ISO). This classification was later modified by pharmacologically using selective agonists and antagonists into α_1 and α_2 plus β_1 and β_2 (Lands *et al.*, 1967). Currently, through molecular cloning, multiple subtypes are now recognized such that α_1-, α_2-, and β-ARs are now each subdivided with a minimum of 3 subtypes. Although found in a wide variety of tissues where they regulate numerous physiological functions, all ARs bind the CAs, ADR and NADR.

1.2.1 Catecholamines

In teleosts, the synthesis and secretion of ADR and NADR occurs within and from specialized cells of the head kidney known as chromaffin cells; these are the teleost homologue of the mammalian adrenal medulla (Wendelaar Bonga, 1997; Fabbri *et al.*, 1998a; Reid *et al.*, 1998). The synthesis occurs through a series of enzymatic reactions termed the "Blaschko pathway" (Randall and Perry, 1992). NADR is produced in vesicles from dopamine by dopamine-β-hydroxylase (DBH). In some cells, NADR then enters the cytosol where it may be methylated into ADR by phenylethanolamine-N-methyl transferase (PNMT). One determinant of the ratio of ADR/NADR produced is the cytosolic presence of PNMT in the cell. However, both hormones are stored within separate storage vesicles until secreted into the blood (Randall and Perry, 1992; Fabbri *et al.*, 1998a; Reid *et al.*, 1998). Basal plasma CA levels in unstressed fish are typically below 10 nM, but within minutes of exposure to a stressor, the levels may range from 4 to 300 nM, and can rise to over 1000 nM with ADR being the dominant hormone released in most teleost species (Gamperl *et al.*,

1994a; Wendelaar Bonga, 1997). The plasma CA levels achieved is dependent upon the type and severity of the stress as well as the species of teleost (Perry and Reid, 1993; Reid *et al.*, 1998).

The biological half-life in the blood is usually less than 10 min because many tissues rapidly take up both CAs (Wendelaar Bonga, 1997). Metabolism of ADR and NADR is catalyzed by the enzymes monoamine oxidase (MAO) and catechol-*o*-methyl transferase (COMT) to produce inactive catabolites (Randall and Perry, 1992; Fabbri *et al.*, 1998a). Activities for these two enzymes have been reported in tissues including the gill, liver and kidney (for references see Randall and Perry, 1992). Given its anatomical location and structure, the gill is believed to be the predominate site for the metabolism of the CAs (Randall and Perry, 1992; Fabbri *et al.*, 1998a). CAs affect cell function through a series of events that are initiated at the cell membrane by binding to specific ARs. This thesis only examines the β-ARs but for completeness both α- and β-AR subtypes will be discussed.

1.2.2 α-Adrenoceptors

Two α-ARs are present in tissues, the α_1- and the α_2-ARs. Molecular and pharmacological studies divide α_1-ARs into α_{1A}, α_{1B}, and α_{1D}- subtypes. Pharmacological evidence for a fourth subtype, termed the α_{1L}, existed but cloning efforts were unsuccessful and this fourth subtype is proposed to be another state of the α_{1A} (Brodde and Michel, 1999). The distribution of the subtypes is tissue specific. For example, the α_{1A}-subtype is found primarily in vas deferens, α_{1B} in liver, and α_{1D} in brain of rats but tissues can express more than one subtype (Milligan *et al.*, 1994; Zhong and Minneman, 1999). There are also species differences; for instance, human liver predominately express the α_{1A}-subtype (Zhong and

Minneman, 1999). The highest degree of homology between the α_1-AR subtypes is observed within the membrane spanning regions with approximately 75% similarity in the amino acid sequence (Ruffolo *et al.*, 1991).

Similarly, studies have subdivided the α_2-ARs into 4 subtypes: α_{2A}, α_{2B}, α_{2C} and α_{2D} (Milligan *et al.*, 1994). However, only three distinct genes have been cloned and it is believed the α_{2A}- and α_{2D}-subtypes may simply represent species-specific pharmacological differences. There is also tissue specific subtype distribution for the α_2-ARs. The α_{2A}-subtype is found primarily on platelets, α_{2B} in kidney, and α_{2C} in brain (Exton, 1985; Milligan *et al.*, 1994). Structurally the α_2-AR subtypes are fairly similar as there is between 70 to 75% amino acid sequence similarity within the membrane spanning regions, and 50 to 60% similarity over the entire length of the sequence (Hein and Kobilka, 1995).

α_1-ARs display pharmacological differences to α_2-ARs in addition to activating a different signal transduction pathway (see section 1.3). The α_1-ARs are preferentially stimulated by NADR and phenylephrine (PHE) and blocked by prazosin (PRZ), while α_2-ARs are more potently stimulated by ADR and clonidine (CLO) and blocked by yohimbine (YOH) (Ruffolo *et al.*, 1991). In addition, α_1-ARs appear to be exclusively post-synaptic with primarily stimulatory effects, while α_2-ARs are found both pre- and post-synaptically and generally demonstrate inhibitory effects (Exton, 1985). α_1-ARs and α_2-ARs are considered as two different groups of ARs, no more related to each other than either is to the β-ARs (Bylund, 1992).

1.2.3 β-Adrenoceptors

In mammals, three different β-ARs have been cloned (Barnes, 1995). Pharmacologically they were originally classified as receptors where ISO acted as an agonist and propranolol (PROP) as an antagonist (Summers and McMartin, 1993). Although all three activate the same signal transduction pathway (see section 1.3), separation into the β_1, β_2, and β_3 types is based on their relative potencies for ADR and NADR. Equal affinity for the two CAs is seen for the β_1-AR, the β_2-AR has higher affinity for ADR and the β_3-AR has a higher affinity for NADR (Ruffolo *et al.*, 1991; Milligan *et al.*, 1994; Lomasney *et al.*, 1995). In terms of sequence similarity, in humans the β_1-AR is 54% homologous to the β_2-AR while the β_3-AR shares only approximately 50% homology with the other two β-ARs (Barnes, 1995). Stronger sequence homology is observed for a given subtype between species and is often in the range of 90% (Mills, 2001). However, certain regions within the third intracellular loop of the three β-ARs are well conserved suggesting all three subtypes couple with the same α-subunit of the G_s-protein (Strosberg, 1993).

Pharmacological evidence has accumulated in recent years to support the possible existence of another β-AR localized to mammalian cardiac tissue that pharmacologically appears distinct from the other three β-ARs, and termed "the putative β_4-AR". This was, in part, based upon activation by the mixed β_1/β_2-antagonist/β_3-agonist CGP 12177 but not by selective β_3-agonists, and the fact that it retained function in β_3-AR knockout mice (Kaumann, 1997; Brodde and Michel, 1999; Dzimiri, 1999). Anatomically, this receptor was found to occur both in atria, ventricles, and the sino-arterial node where it promotes increases in Ca^{2+} transients through coupling to G_s (Dzimiri, 1999; Sarsero *et al.*, 1999). More recent evidence, however, suggests that this receptor is simply a novel state of the β_1-

AR (Granneman, 2001). This latter example indicates that our knowledge of the AR gene family remains far from complete.

1.3 Signal transduction

Both ADR and NADR are hydrophilic and therefore must bind to a cell surface receptor in order to elicit a physiological response. The ligand-binding pocket is found within the seven membrane spanning domains of the ARs that are believed to be α helices (Strosberg, 1993; Lomasney *et al.*, 1995). Agonist binding occurs approximately one-third of the way within the membrane core and involves interactions with specific amino acids. While other amino acids may be involved in binding, aspartate (Asp) 113 in the third transmembrane domain and serine (Ser) 204 and 207 in the fifth domain occur in homologous positions in all ARs and are considered key to binding (Strosberg, 1993; Johnson, 1998). Upon binding of either one of the CAs or another agonist, conformational changes in the receptor are induced permitting G-protein interaction (Raymond, 1995). Specific amino acids contained in the third intracellular loop between membrane spanning domains 5 and 6 are responsible for coupling to the carboxy-terminal domain of the α-subunit of the respective G-protein (Strosberg, 1993; Lomasney *et al.*, 1995; Raymond, 1995).

G-proteins or GTP-binding proteins are a group of membrane bound heterotrimeric proteins consisting of α, β, and γ subunits (Duman and Nestler, 1995). There are four major types of G-proteins called G_s, $G_{i/o}$, $G_{q/11}$, and $G_{12/13}$ families. However, the α-subunit itself belongs to a larger family of guanosine triphosphatases (GTPases) that includes "small" G-proteins such as ras (Raymond, 1995). Interaction with an agonist bound receptor results in

an exchange of GDP for GTP and the reversible dissociation of the βγ dimer from the α-GTP subunit which then activates an effector molecule resulting in second messenger formation (Lohse *et al.*, 1996). Intrinsic GTPase activity in the α-subunit converts the α-GTP complex into α-GDP, which possesses a high affinity for the βγ dimer and the trimeric G-protein re-associates (Birnbaumer and Birnbaumer, 1995). In addition, apart from stabilizing interactions between the α-subunit and receptors, membranes and effector enzymes, the βγ dimer may also serve as a signaling molecule (Raymond, 1995). The β-subunit can become phosphorylated resulting in activation of ACase (Birnbaumer and Birnbaumer, 1995). As well, in mammalian brain, where the α_q-subunit activates phospholipase C-β (PLC-β1), the βγ dimer is found to stimulate PLC-β2 (Duman and Nestler, 1995). A recent study reports the βγ subunits also regulate membrane Ca^{2+} channels (Wolfe *et al.*, 2003).

Activation of the α_1-ARs by an agonist, through the G_q-protein stimulates PLC-β resulting in the formation of two second messengers, membrane bound 1,2-diacylgyerol (DAG) which activates protein kinase C (PKC), and inositol 1,4,5-trisphosphate (IP_3) which in the cytosol binds to specific intracellular receptors that release sequestered Ca^{2+} from intracellular stores (Summers and McMartin, 1993).

The α_2-ARs couple to a G-inhibiting or G_i protein that inhibits the activity of the membrane-bound adenylyl cyclase (ACase) leading to a reduction in the intracellular levels of cyclic AMP (cAMP). As well, these receptors can operate by directly modifying ion channel activities such as the K^+ channel, Ca^{2+} channel, and the Na^+/H^+ antiport exchanger (Summers and McMartin, 1993).

All β-ARs are coupled to the G_s or G-stimulating protein that activates ACase that catalyzes ATP to form the second messenger cAMP. Intracellular levels of cAMP which

may increase up to 400-fold, in turn activate protein kinase A (PKA) (Exton, 1985; Lomasney *et al.*, 1995). In the inactive state, PKA is a tetramer consisting of two regulatory and two catalytic subunits. Binding of cAMP to the regulatory subunits results in the release of the active catalytic subunits that then phosphorylate specific proteins to produce a cellular response (Marullo *et al.*, 1995). A schematic representation of the three signal transduction pathways in a liver cell is presented in Figure 1.2.

1.4 β-Adrenoceptor regulation

In order to maintain the response of the cell within physiological limits, agonist exposure also leads to a decrease in receptor responsiveness by a complex regulatory process collectively known as desensitization (Lohse, 1993). This reduced response may be the result of a combination of different processes, including receptor phosphorylation leading to an uncoupling of the receptor from the G-protein, receptor internalization from the plasma membrane to intracellular compartments, and the reduction in cell receptor number due to decreased receptor mRNA and protein synthesis as well as degradation of pre-existing receptors (Ferguson, 2001). Two major categories of desensitization are recognized. The first is homologous or agonist-specific desensitization whereby the response of the cell is diminished to the acting agonist or that class of agonists with no change in responsiveness to other agonists. Medina-Martínez and García-Sáinz (1993) showed in isolated hypothyroid rat hepatocytes that a 15 min incubation with 10 μM ISO diminished β-AR but not glucagon-stimulated cAMP production. In contrast, the second type is heterologous or generalized desensitization whereby the decreased responsiveness is observed not only to the acting

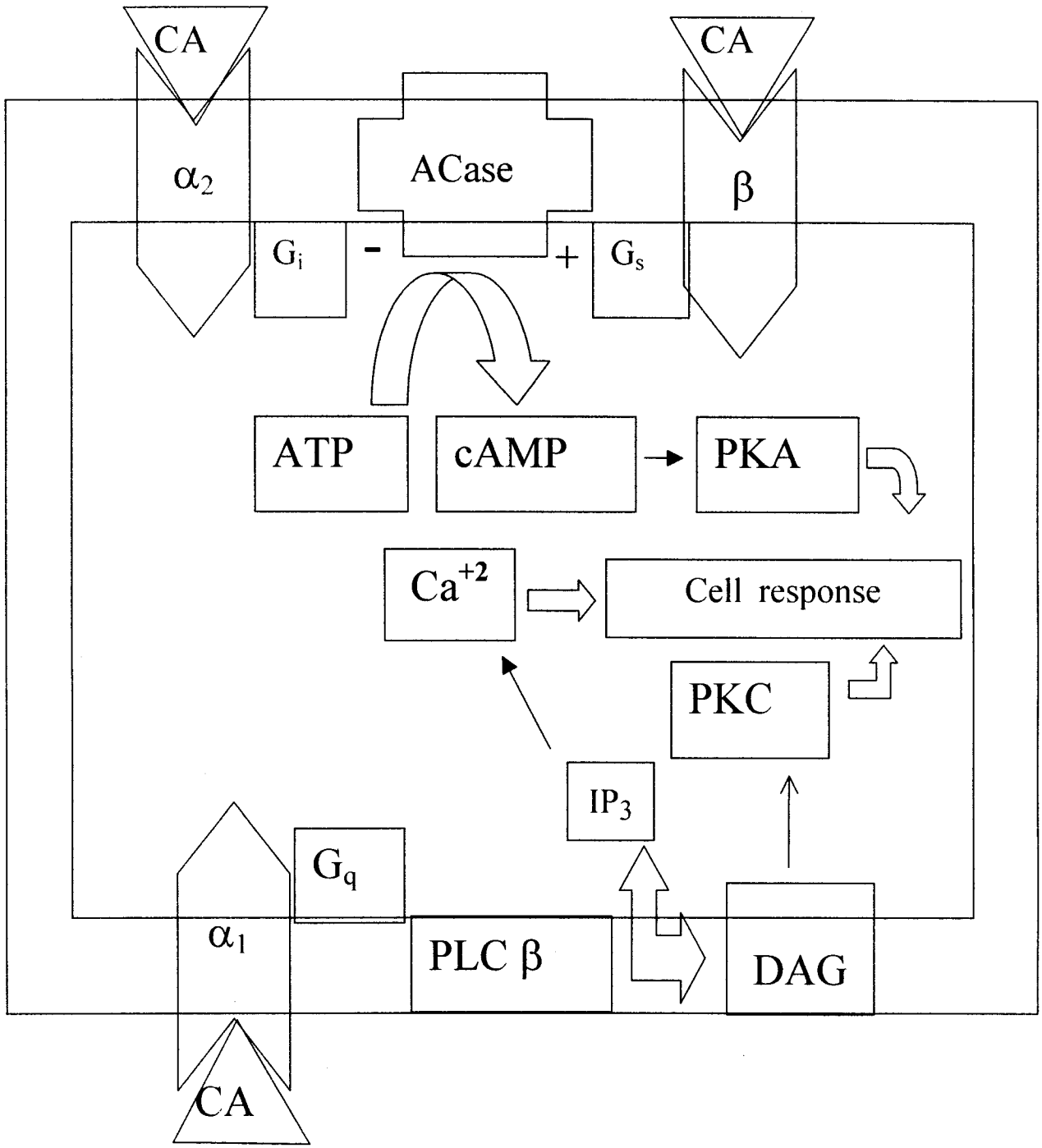


Figure 1.2. Adrenergic signal transduction pathways in a liver cell. See text for details. Figure modified from Fabbri *et al.* (1998a).

agonist but also to a variety of other agonists that activate the same signaling pathway (Lefkowitz and Caron, 1986; Lohse, 1993; Fabbri and Moon, 1994). Hernández-Sotomayor *et al.* (1988) found that incubating isolated hepatocytes from hypothyroid rats with the PKC activator phorbol myristate acetate (0.1 μM) for 15 min significantly diminished both ISO and glucagon-stimulated cAMP production. The extent of the desensitization incurred has been found to be dependent upon the duration and degree of agonist exposure (Johnson, 1998).

Agonist occupied ARs are phosphorylated by a member of the family of G-protein receptor kinases (GRK) called β-adrenergic kinase (βARK1 or GRK2). Phosphorylation initiates a process that ultimately uncouples the receptor from the G-protein. The βγ-dimer of the G-protein may act as an anchor for cytosolic βARK to translocate to the membrane to phosphorylate the plasma membrane bound AR (Barnes, 1995; Lohse *et al.*, 1996). The uncoupling of the G-protein from the AR is a result of the binding of another protein, termed β-arrestin, to the phosphorylated receptor. In addition to its uncoupling function, β-arrestin may also act as an adaptor protein aiding in the internalization of the phosphorylated receptor into a clathrin-coated vesicle (Ferguson, 2001). βARK1 phosphorylates carboxy-terminal serine and threonine residues and is believed to be responsible for homologous desensitization (Hein and Kobilka, 1995). Unoccupied receptors can be phosphorylated by either PKA or PKC resulting in heterologous desensitization; receptor function is directly impaired as no accessory proteins such as β-arrestin are required (Hein and Kobilka, 1995). Both PKA and PKC phosphorylate serine residues on both the third intracellular loop and C-terminal domain of the mammalian AR (Summers and McMartin, 1993; Ferguson, 2001).

However, the third intracellular loop appears to be the preferred site. Phosphorylation in this region impairs the ability of the β-AR to interact with the G_s-protein (Lohse, 1993).

Prolonged agonist exposure may result in the loss of cell surface receptors. This loss is divided into two phenomena, sequestration and down-regulation. Sequestration is a result of cell surface ARs being translocated into intracellular compartments and is rapidly reversible (within minutes) upon removal of the agonist (Hein and Kobilka, 1995). Although slower than uncoupling, sequestration is still a relatively rapid process as depending upon the cell type, 30 to 50% of β_2-ARs may become internalized after only 10-20 min of agonist exposure (Lomasney *et al.*, 1995). The internalized receptors are inaccessible to hydrophilic ligands but are still accessible to lipophilic ligands, which can penetrate vesicular membranes (Duman and Nestler, 1995; Lomasney *et al.*, 1995). In addition, sequestration may serve an important role in receptor resensitization as dephosphorylation of the receptor occurs within the intracellular compartments (Hein and Kobilka, 1995; Johnson, 1998).

Down-regulation refers to an actual decrease in the total number of receptors in the cell and a return to basal AR levels requires receptor biosynthesis (Barnes, 1995; Bousquet-Mélou *et al.*, 1995). This loss is a result of both a decrease in receptor synthesis due to a cAMP-dependent destabilization in receptor mRNA and a proteolytic degradation of receptors in lysosomes. Down-regulation of the mRNA may be due to an up-regulation of a RNA binding protein that reduces receptor mRNA stability (Wallukat, 2002). As such, down-regulation is a much slower developing process than desensitization or sequestration, requiring hours to days (Lomasney *et al.*, 1995; Wallukat, 2002). The processes of densitization and down-regulation are presented diagrammatically in Figure 1.3.

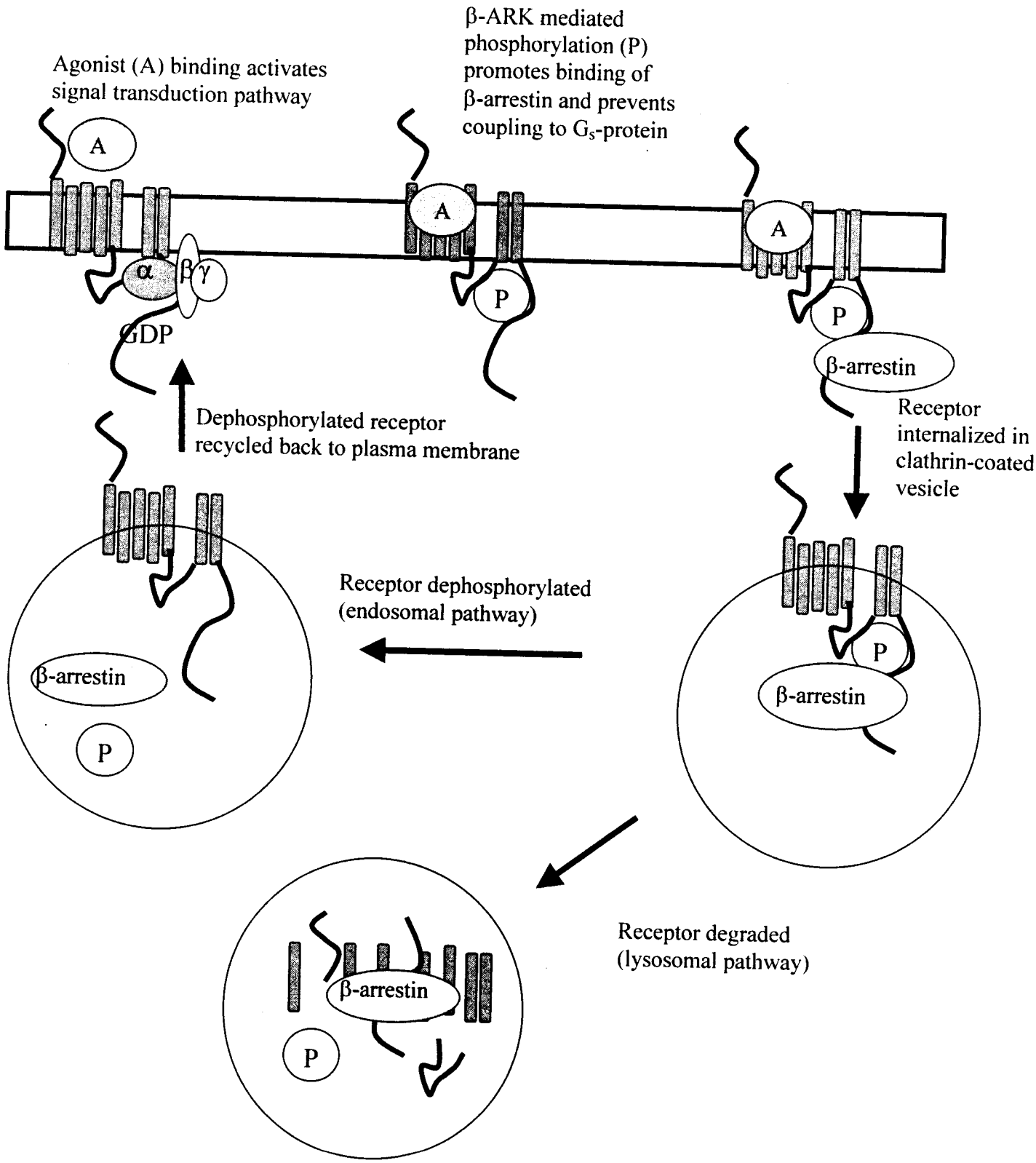


Figure 1.3. Schematic representation of the mammalian model of β-AR desensitization and down-regulation. See details in text. Figure modified from McDonald and Lefkowitz (2001).

The three β-AR subtypes in mammals display different sensitivities in their responsiveness to desensitization. Of the three, the β_2-AR is the best characterized and exhibits a high degree of desensitization compared with the other β-AR subtypes (Lohse, 1993). The β_1-AR possesses fewer potential phosphorylation sites that are known to be important in the desensitization of the β_2-AR (Marullo *et al.*, 1995). Compared with the other two β-ARs, the β_3-AR has a short intracellular tail containing few potential phosphorylation sites and is almost completely resistant to desensitization (Summers and McMartin, 1993; Marullo *et al.*, 1995).

1.5 Mammalian β-adrenoceptors

The physiological roles played by ARs in ameliorating the affects of a stressor are both subtype and tissue specific. In mammals, the three β-AR subtypes are distributed in numerous tissues throughout the body, frequently with more than one subtype being found within a particular tissue. In fact, β_1- and β_2-ARs are often co-expressed, however, the ratios of each are tissue specific (Mills, 2001). β_1-ARs are the dominant β-AR subtype in the heart and can also be found in such tissues as adipose, lung, and skeletal muscle. β_2-ARs have been characterized in a number of tissues including heart, liver, lung, pancreas, salivary glands, as well as smooth and skeletal muscles (Exton, 1985; Mills, 2001). Of the three β-ARs, β_3-ARs show the smallest distribution pattern being restricted primarily to adipose tissues (Mills, 2001).

Much research in mammalian β-ARs has been directed at the receptors in cardiac tissue, especially their regulation during periods of heart failure. All three of the cloned β-

ARs exist in human cardiac tissue with the β_1- and β_2-subtypes being the most prominent (Milligan *et al.*, 1994). In both atrial and ventricular tissues, the β_1-subtype outnumbers the β_2-AR by approximately a 2:1 ratio or more. Stimulation of these receptors results in enhanced strength (inotropic) and frequency (chronotropic) of contractions (Brodde and Michel, 1999; Dzimiri, 1999). Activation of either β_1- or β_2-ARs through G_s results in PKA-mediated phosphorylation of such sarcolemmal proteins as the L-type Ca^{2+} channel and phospholamban. The former enhances contraction through promotion of Ca^{2+} influx, while the latter increases Ca^{2+} uptake into the sarcoplasmic reticulum to enhance diastolic relaxation (Brodde and Michel, 1999).

Although β_1-ARs are the predominant subtype in human cardiac tissue, functionally the responses by the two subtypes may not be different as a result of a more efficient coupling of the β_2-subtype to ACase (Harding *et al.*, 1994; Brodde and Michel, 1999). In addition, β_2-ARs are found in high proportions in the pacemaker and conducting regions of the heart where they play a key role in controlling both heart rate and rhythm (Dzimiri, 1999).

While the co-existence of the β_1- and β_2-subtypes in myocardial tissue is undeniable, the possible existence of a third and potentially a fourth cardiac β-AR is equivocal. Molecular studies are mixed for the existence of the β_3-AR in human cardiac tissue (for references see Brodde and Michel, 1999).

Functionally, in isolated human ventricular tissue selective β_3-agonists demonstrate cardio-depressant effects and shortening of the action potential (Kaumann, 1997). These effects are thought to be mediated through the β_3-AR coupling to the G_i- protein (Kaumann, 1997; Dzimiri, 1999). Conversely, β_3-ARs are capable of promoting positive inotropic

effects in isolated atrial tissue (Dzimiri, 1999). As well, CGP 12177, which is an antagonist on the β_1- and β_2-subtypes but a β_3-agonist, also demonstrates positive inotropic effects in human atrial tissue, suggesting coupling to G_s (Brodde and Michel, 1999). Clearly, more research is needed to determine more precisely what role, if any the β_3-AR has in regulating cardiac function (Brodde and Michel, 1999; Dzimiri, 1999).

ARs are also involved in regulating various metabolic functions. In stressful situations, CAs directly stimulate carbohydrate metabolism in liver and muscle and indirectly in adipose tissue. In the liver, ARs increase blood glucose levels by activating glycogenolysis and gluconeogenesis. CA-mediated lipolysis in adipose tissue provides the liver with glycerol for gluconeogenesis (Chu *et al.*, 2000). In most mammalian species studied, hepatic α_1-ARs are more highly expressed than β-ARs (Sulakhe *et al.*, 1988) and as such CA-stimulated glucose production is primarily mediated by α_1-ARs although in humans it is mediated predominantly by β_2-ARs (Exton, 1985). However, this α_1-AR dominance is only observed in the adult, as β-ARs are the predominant hepatic AR found in fetal and neonatal mammals (Sulakhe *et al.*, 1988; Rao *et al.*, 1995). During development, the dependence on circulating CAs from the adrenomedulla is replaced by synaptic release of NADR (Rao *et al.*, 1995). In the adult, sympathetic nerve terminals directly innervate liver parenchymal cells; however there is significant species variation in the degree of this innervation (Sulakhe *et al.*, 1988). In addition, it has been shown *in vitro* that in the liver, NADR functions through α_1-ARs while ADR works through the β_2-ARs (Chu *et al.*, 2000).

Adipocytes and brown adipose tissue (BAT) possess all three β-AR subtypes where they regulate lipolysis and thermogenesis, respectively (D'Allaire *et al.*, 1995; Mills, 2001; Mills *et al.*, 2003a). Stimulation of the ACase/cAMP signal transduction pathway in

adipocytes results in the activation of hormone-sensitive lipase. The lipolytic actions of CAs in adipose tissue are mediated primarily through β_3-ARs although there are species-specific differences. For example, while β_3-ARs are the primary mediator of lipolysis in rodents, a predominantly β_1-AR-mediated response is found in pigs (Mills *et al.*, 2003a). Similar to the lipolytic response, NADR-stimulated BAT thermogenesis in rodents is predominantly mediated by β_3-ARs (D'Allaire *et al.*, 1995; Zhao *et al.*, 1998).

1.6 β-Adrenoceptors in teleosts

Catecholamines are secreted into the circulation of fish in response to a number of stressors including hypoxia, exhaustive exercise, and air exposure. The end result of CA release is cardiorespiratory modifications as well as mobilization of energy reserves designed to ameliorate the deleterious effects of the stressor (Perry and Reid, 1993; Fabbri *et al.*, 1998a; Reid *et al.*, 1998). As in mammals, β-ARs in teleosts show the same structure of seven membrane spanning domains, an extracellular amino head and an intracellular carboxy terminus (Nickerson *et al.*, 2001). In addition, the signal transduction pathway activated appears to be the same G_s/ACase coupled system seen in mammals (Fabbri *et al.*, 1998a). The α- and β-subunits of G_s have been identified in the olfactory epithelium of the channel catfish, *Ictalurus punctatus* (Abogadie *et al.*, 1995). Recently, using black bullhead hepatic membranes, Fabbri *et al.* (2002) provided the first direct evidence for the presence of G_s α-subunits in the liver from a lower branching vertebrate. Activation of ACase leading to cAMP production is well demonstrated in a number of tissues (gill, Guibbolini and Lahlou, 1987; liver, Ottolenghi *et al.*, 1988; Fabbri *et al.*, 1992 (black bullhead); 1995a (rainbow

trout); 1998b (European eel, *Anguilla anguilla*); red blood cell, Reid *et al.*, 1993; red and white muscles, Lortie and Moon, 2003).

Studies of β-ARs in teleosts have been limited to relatively few species. Of those species studied, the best characterized species by far is the rainbow trout. Tissues in this species where β-ARs are characterized include liver (Reid *et al.*, 1992; Fabbri *et al.*, 1995a; Dugan and Moon, 1998; Nickerson *et al.*, 2001), heart (Keen *et al.*, 1993; Gamperl *et al.*, 1994b; Olsson *et al.*, 2000), red and white muscles (Lortie and Moon, 2003), and red blood cells (Perry and Reid, 1992; Gilmour *et al.*, 1994; Reid and Perry, 1995; Nickerson *et al.*, 2003). As in mammals, β-ARs in teleosts are widely distributed throughout the body and have been characterized in a variety of other tissues including gill and brain of brown bullhead, *A. nebulosus* (Steevens *et al.*, 1996), head kidney, spleen and peritoneal cells of goldfish, *Carassius auratus* (Jozefowski and Plytycz, 1998), leukocytes of channel catfish (Finkenbine *et al.*, 2002), and adipocytes of carp, *Cyprinus carpio* (Van den Thillart *et al.*, 2001) and tilapia, *Oreochromis mossambicus* (Vianen *et al.*, 2002).

While molecular biology and phylogenetic analysis supports the existence of three distinct AR subtypes in rainbow trout (Nickerson *et al.*, 2001; 2003), pharmacological classification of teleost β-ARs into distinct subtypes is difficult as currently only mammalian-specific agonists and antagonists are available. While agonists and antagonists may be selective for a particular subtype in mammals, such selectivity is not necessarily found in other groups of vertebrates. For instance, phenylephrine is a selective mammalian α_1-AR agonist but acts as an agonist on the hepatic β-AR in teleosts (Brighenti *et al.*, 1987a,b; Moon and Mommsen, 1990). In fact, even in mammals there are species-specific discrepancies in subtype selectivity for some ligands. For example, BRL 37344 is a selective

β_3-AR agonist in rats but is selective for the β_2-AR of pigs (Liang and Mills, 2001). The affinity a ligand has for a receptor subtype is strongly dependent upon the amino acid sequence of the receptor, which varies between species (Mills, 2001). However, insight into the physiological function of specific subtypes can be provided through the use of transfection studies. Still some caution must be exercised as expression levels of the endogenous receptor frequently occurs at much lower levels and as such at the quantitative level, there may be different functional consequences (Brodde and Michel, 1999).

In the teleost liver, β-ARs are involved in the mobilization of glucose primarily by glycogenolysis through PKA activation of glycogen phosphorylase (GPase) (Perry and Reid 1993; Fabbri and Moon, 1994; Moon *et al.*, 1999). CA-induced hyperglycemia has been demonstrated in numerous fish species *in vivo* and *in vitro* (for references see Fabbri *et al.*, 1998a). The hepatic β-AR has been pharmacologically characterized in rainbow trout (Reid *et al.*, 1992; Nickerson *et al.*, 2001) and European eel (Fabbri *et al.*, 2001) as most closely resembling the mammalian β_2-AR subtype. This is in agreement with the subtype found in the liver of other lower branching vertebrates including the Australian lungfish (*Neoceratodus forsteri*), the axolotl (*Ambystoma mexicanum*), and the toad (*Xenopus laevis*) (Janssens and Grigg, 1988). As well, the β_2-AR has been shown to be the predominant hepatic β-AR subtype in a number of mammalian species including the rat (*Rat norvegicus*) (Wolfe *et al.*, 1976), the human (*Homo sapiens*) (Kawai *et al.*, 1986), and the dog (*Canis familiaris*) (García-Sàinz *et al.*, 1996).

Of the three subtypes cloned in rainbow trout, the β_2-AR subtype was shown to be the most widely distributed. While the highest levels were found in liver and red muscle, expression was also observed in white muscle, spleen, kidney, gill and heart (Nickerson *et*

al., 2001). The other two subtypes group phylogenetically with the mammalian β_3-ARs and are termed β_{3a}- and β_{3b}-ARs. Highest levels for the β_{3a}-AR were shown in heart and gills with expression also found in kidney and white muscle (Nickerson *et al.*, 2003).

A novel role for β-ARs is found in the red blood cell of some teleosts. CA binding results in the activation of a cAMP-dependent membrane bound Na^+/H^+ exchanger. The role of this exchanger is to extrude H^+ from the cell with the resulting intracellular alkalinization enhancing haemoglobin O_2 binding affinity and thus increasing the O_2 carrying capacity of the blood. However, significant species differences were observed with salmonids showing the greatest response of all the groups of teleosts studied to date (Perry and Reid, 1993; Wendelaar Bonga, 1997). The specific subtype present on the trout red cell was demonstrated pharmacologically to be a β_1-AR (Tetens *et al.*, 1988). More recently, Nickerson *et al.* (2003) used molecular evidence to establish that the β_{3b}-AR is exclusively localized to the red blood cell of the rainbow trout.

As described in section 1.4, phosphorylation at specific sites plays an important role in receptor regulation. As seen with mammalian β-ARs, the three rainbow trout β-ARs display different potential phosphorylation profiles and thus may display different patterns of regulation. The trout β_2-AR possesses potential phosphorylation sites in the third intracellular loop however none are in the G-protein binding domain. There were three potential phosphorylation sites found in the carboxy tail with only one that aligned with a similar site in the mammalian β_2-AR (Nickerson *et al.*, 2001). More potential phosphorylation sites are found in the rainbow trout β_{3a}- and β_{3b}-ARs. The β_{3a}-AR has 8 and 2 potential sites, while the β_{3b}-AR was found to have 6 and 8 potential sites in the 3rd intracellular loop and cytoplasmic tail, respectively (Nickerson, *et al.*, 2004). Given the

differences in the number of potential phosphorylation sites, it is highly probable the same variability in the sensitivity to desensitization and down-regulation observed within mammalian β-ARs, will also be found in the different trout β-ARs.

Studies on the regulation of β-ARs in teleosts are limited. To date, only two studies have shown significant decreases in β-ARs. Gilmour *et al.* (1994) showed a 33% decrease in rainbow trout red blood cell β-ARs after 3 d of chronic CA exposure using mini-osmotic pumps. Also in red blood cells, Perry *et al.* (1996) found 20% and 30% decreases in β-ARs under normoxic and hypoxic conditions, respectively, after 7 d of daily chasing. A β-arrestin was cloned in trout red blood cells (Jahns *et al.*, 1996), and desensitization in trout red blood cells after 48 h of moderate hypoxia is reported (Thomas *et al.*, 1991). Detectable levels of β-arrestin and β-ARK mRNA were found in rainbow trout liver but to a lesser extent than in red blood cells (Nickerson *et al.*, 2004).

Therefore, the objective of this thesis was to examine and provide insight into the regulation of β-ARs in teleosts. This was accomplished by comparing how stressors affect the distribution and function of β-ARs in the same tissue (liver) from two different species of teleosts, the rainbow trout and black bullhead. As well, how stressor exposure affects the properties of both the β_2- and β_{3a}-ARs in two different tissues (liver and gill) from the same species, the rainbow trout.

Chapter 2: β_2-adrenoceptor regulation in rainbow trout liver

Adapted from Dugan, S.G., Lortie, M.B., Nickerson, J.G., and Moon, T.W. (2003) *Comp. Biochem. Physiol.* **136B**:331-342.

2.1 Introduction

Hormone receptors during continuous agonist exposure generally display decreased responsiveness to further stimulation by a process termed desensitization. This rapid process occurs within minutes of agonist exposure. Should exposures continue for hours or days, receptors may become internalized (sequestration) where they may be either recycled back to the cell surface or degraded resulting in a decrease in the total number of receptors, termed down-regulation. These processes are well defined in the large superfamily of receptors that function through coupling to G-proteins, especially the receptors that bind the catecholamines adrenaline (ADR) and noradrenaline (NADR), termed adrenergic receptors or adrenoceptors (AR) (Lohse, 1993; Barnes 1995).

In teleost fishes, ADR and NADR are released into the circulation after exposure to some form of stressor resulting in cardiorespiratory modifications and mobilization of energy reserves (Fabbri *et al.*, 1998a; Reid *et al.*, 1998). As in mammals, binding of ADR and NADR to the β-AR results in activation of adenylyl cyclase (ACase) leading to increased intracellular cyclic AMP (cAMP) levels. Previous characterization of the hepatic β-ARs in the rainbow trout, *Oncorhynchus mykiss*, supports the presence of a single class of binding sites (Fabbri *et al.*, 1995a; Dugan and Moon, 1998) that are pharmacologically most similar to the mammalian β_2-AR (Reid *et al.*, 1992; Nickerson *et al.*, 2001). This pharmacological classification is supported by phylogenetic analysis of cDNA clones that identified only a β_2-AR homologue in trout liver (Nickerson *et al.*, 2001). Although trout tissues also express a

β-AR homologous to the mammalian β_3-AR, this form is not found in the liver using a gene specific RNA protection assay (Nickerson *et al.*, 2003).

Although many studies have characterized the distribution and function of different β-AR subtypes in teleosts (for references see Fabbri *et al.*, 1998a; Reid *et al.*, 1998), few have examined their agonist regulation. Increased circulating catecholamine levels induced by either physical stress (Perry *et al.*, 1996) or chronic administration (Gilmour *et al.*, 1994) decreased the numbers of red blood cell surface β-ARs, a receptor now classified as a β_{3b}-AR (Nickerson *et al.*, 2003). So while agonist regulation is well studied for mammalian β_2-ARs, whether agonist regulation of the β_2-AR occurs in teleosts is not currently known. This study tests the hypothesis that regulation of the hepatic β_2-AR in rainbow trout involves a loss of binding sites from the cell surface. To test this hypothesis, I examined the distribution and signal transduction pathway of the trout hepatic β_2-AR as affected by chasing to exhaustion, a physical stressor known to increase plasma catecholamines (Perry *et al.*, 1996), and by prolonged exposure to clenbuterol (CLEN) and ractopamine (RACT), two agonists that act on mammalian β-ARs (Mills, 2002) and that are used as repartitioning agents in agriculturally important animals, including fish (Vanderbergh and Moccia, 1998).

2.2 Materials and methods

2.2.1 Animals

Female rainbow trout weighing approximately 80-200 g were obtained from Linwood Acres Trout Farm (Campbellcroft, ON). Fish were transported to the University of Ottawa where they were acclimated for a period of at least 6 weeks in fibreglass holding tanks (1275 L) of well aerated, dechloraminated city of Ottawa tap water at 13.0 ± 1.0°C with a constant

12L:12D photoperiod. Fish were fed 5 times a week with commercial trout pellets (Martin Mills). Physical stress, agonist feeding and cAMP production studies were conducted from May to July, while agonist competition studies were conducted in January and February. Trout were cared for and treated in accordance with the guidelines from the Canadian Council on Animal Care.

2.2.2 Experimental protocol

Each chasing experiment used 12 fish (approximate weight 200 g) removed from the main holding tank, divided into two groups of 6 and placed in 115 L tanks 1 week prior to experimentation. One group served as a control while the second group was physically stressed using the chasing protocol of Perry *et al.* (1996). Individuals from this latter tank were removed to a small bucket and chased until they could be handled without resistance (approximately 5-7 min) once per day for up to 7d. Two fish from each tank were sampled one-hour post chasing after 1, 3 and 7 d. One hour was selected as catecholamine levels would be elevated (Perry *et al.*, 1996) and changes in receptor characteristics should have occurred by that time. Non-chased controls were randomly selected from the second holding tank. Fish were killed by a quick blow to the head between the hours of 12 to 2 pm and a blood sample rapidly withdrawn from the caudal vessel into a heparinized syringe. Blood was centrifuged for 1 min (12,000 g; Eppendorf 5415 C), the plasma removed, frozen in liquid nitrogen and stored at -70°C for future analysis. Livers were quickly removed and processed individually for crude membrane preparations. This protocol was repeated three times over a 4 week period resulting in six control and six chased fish at the 1, 3, and 7 d interval.

Agonist feeding experiments were performed according to Lortie *et al.* (2004). Briefly, three groups of 20 fish (approximate weight 80 g) were placed in separate 115 L tanks. Trout were fed commercial trout pellets (Martin Mills 5 PT, size 5 mm) twice daily for 37 days (total of 1.5% body weight per day) to which was added 40 ppm CLEN or RACT or the ethanol carrier (SHAM). CLEN was obtained from Sigma Chemical Co. while RACT was a generous gift from Eli Lilly (Greenfield, IN). The diastereoisomer ratio of RACT was RS, SR = 51.02%; RR, SS = 48.98%. On the 37^{th} day, fish were sacrificed by a quick blow to the head 4 h after the morning feeding, livers were removed and freeze-clamped in liquid nitrogen and stored at -70°C. Before freezing, a small sample of liver (approximately 500 mg) was removed and frozen for β_2-AR mRNA analysis. Blood was sampled as described for the chasing study.

2.2.3 Membrane preparations

Crude membranes were prepared from either fresh (chasing study) or frozen (agonist feeding study) livers. Frozen livers were ground individually in a mortar and pestle under liquid nitrogen prior to homogenization. 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 $MgSO_4$, 0.33 NaH_2PO_4, 0.44 KH_2PO_4, 5.0 Hepes, 5.0 Hepes-Na, and 1.0 $NaHCO_3$, pH 7.63), resuspended in 8 volumes of homogenization buffer (in mM: 0.8 $MgCl_2$, 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 $MgCl_2$, 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. Protein content for all liver membrane preparations was determined using the bicinchoninic acid (BCA) assay (Sigma) with bovine serum albumin (BSA) as standard.

2.2.4 Binding assays

Binding to hepatic membrane β-ARs was determined using the hydrophilic β-antagonist ((-)-4-(3-t-butylamino-2-hydroxypropoxy)-[5,7-^{3}H]benzimidazol-2-one) ^{3}H-CGP-12177 (^{3}H-CGP; Amersham, specific activity 46.0 Ci/mmol). Preliminary studies showed no differences in binding between fresh (chasing study) and frozen (agonist feeding study) membrane preparations. Fifty µl of membranes (containing 220 - 325 µg protein, depending upon the study) were incubated for 60 min at room temperature (19 ± 1°C) in a final volume of 150 µl in the presence of varying concentrations of ^{3}H-CGP. Non-specific binding was determined in the presence of 10 µM alprenolol (ALP). Specific binding was the difference between total and non-specific binding. To characterize the hepatic β-AR or to determine the inhibitory constants (K_i) of CLEN and RACT, crude membranes were prepared as above from frozen livers sampled from a separate group of trout in the main holding tank. Fifty µl of crude membranes were incubated with a saturating concentration of ^{3}H-CGP (approximately 1 nM) alone or with increasing concentrations (ranging from 1nM to 10 µM) of selective and non-selective agonists and antagonists or with CLEN and RACT (10 nM to 100 µM) for 1 h at room temperature. In order to prevent photodegradation, incubations

were done in the dark and aluminum foil was used to cover the microcentrifuge tubes containing the ligands. Sixty minutes was chosen based on preliminary experiments showing linearity in binding up to 90 min. All incubations were terminated with 4 washes of ice-cold 0.9% NaCl using a cell harvester (Brandel 24R). Membranes were collected on borosilicate filters (#32 Mandel Scientific) and placed in scintillation vials containing 4 ml scintillation cocktail (Safety-Solve; RPI, Mount Prospect, IL). Vials were left in the dark at room temperature for at least 24 h before radioactivity was determined using either a Packard 2500 TR or a Beckman Coulter LS 6500 liquid scintillation counter. Binding parameters (K_d, K_i, and B_{max}) were determined using the EBDA and LIGAND computer programs (Munson and Rodbard, 1980).

2.2.5 Adenylyl cyclase (ACase)

Adenylyl cyclase (ACase) activities were assessed using a modification of the method of Fabbri *et al.* (1998b). To maximize possible differences in ACase activites, membranes were selected from the three SHAM fed fish that displayed the highest number of ^{3}H-CGP saturable binding sites while the three CLEN fed fish that displayed the lowest number of binding sites were selected. One hundred microlitres of membranes (containing approximately 150 µg of protein) were incubated in a final volume of 400 µl containing (in mM) 100 Tris-HCl, 5 $MgCl_2$, 1 ATP, $1x10^{-3}$ GTP, 5 theophylline, pH 7.4, and the agonists (10^{-4} M isoproterenol (ISO), 10^{-2} M NaF, 10^{-5} M forskolin (FSK)). The assay consisted of a 10 min incubation at 25°C after the addition of ATP followed by termination of the reaction in boiling water for 2 min. Samples were then frozen at -20°C for later analysis. Upon

thawing, samples were centrifuged at 4,000 g for 10 min at 4°C. cAMP was determined in supernatants using a commercial enzyme immunoassay (EIA) kit (Amersham).

2.2.6 β_2-AR mRNA Isolation and assay

Trout liver RNA from agonist fed fish was isolated using Trizol reagent (Gibco BRL, Burlington, Ontario) and RNA quality and quantity were verified by gel electrophoresis and spectrophotometry. RNA used for RNase protection assays was treated with the DNA Free™ kit (Ambion) as described by Nickerson *et al.* (2001).

Trout liver β_2-AR mRNA levels from SHAM and CLEN-fed fish were measured using the semi-quantitative RNase protection assay RPA III™ (Ambion) and gene specific probes for trout β_2-AR and trout β-actin as described in Nickerson *et al.* (2001). These gene specific probes span the third intracellular loop and are designed to distinguish between the β_2-AR mRNA and the other β-AR mRNAs in trout (Nickerson *et al.*, 2003).

Expression levels for trout liver β_2-AR and β-actin mRNAs 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_2-AR and β-actin bands were measured using the Quantity One software package (version 4.0.3, BioRad). β_2-AR expression levels for SHAM and CLEN samples were expressed as the ratio of β_2-AR band density versus β-actin band density. The mean ratio of β_2-AR band density to β-actin band density was calculated for SHAM and CLEN groups and these mean ratios were compared using the Mann-Whitney rank sum test (Sigma Stat, version 2.03, SPSS Inc.).

2.2.7 Hepatocyte isolation

Hepatocytes were isolated using the method of Mommsen *et al.* (1994) to examine the ability of CLEN and RACT to act as agonists on rainbow trout hepatic β_2-AR function. Fish were randomly sampled from the main holding tank and killed by a quick blow to the head. The hepatic portal vein was cannulated and the liver was rinsed at a flow rate of approximately 2 ml/min for 5 to 10 min with Hanks' buffered saline containing 1.0 mM EGTA (pH 7.63). The perfusion was continued for an additional 15-20 min with the same medium in the absence of EGTA, but supplemented with collagenase (150 mg/ml; Sigma type IV). The liver was then excised, minced with a razor blade and filtered through two nylon screens (253 and 73 μm, respectively). Hepatocytes were washed 4-times by centrifugation (Sorvall® RC 5B Plus) at 120 g for 2 min. The first two rinses were in Hanks' medium without EGTA and the last two in Hanks' supplemented with 1.5 mM $CaCl_2$ and 2% BSA. After the last wash, hepatocytes were allowed a 1 h metabolic rest on ice; hepatocytes were then resuspended in fresh medium to a concentration of 80 to 100 mg/ml. Viability was verified by trypan blue exclusion and was greater than 80% in all experiments.

2.2.8 cAMP estimates

Approximately 8 to 10 mg of hepatocytes were incubated for 15 min at room temperature with either Hanks' medium (15'CTL), 10^{-7}M ISO, 10^{-5}M CLEN, or 10^{-5}M RACT alone or in combination with ISO, or 10^{-5}M FSK. In the combination experiments, cells were preincubated for 10 min with either CLEN or RACT before the addition of ISO. All agonists were purchased from Sigma (St. Louis, MO) except for RACT (Eli-Lily; Greenfield, IN). Assays were terminated with the addition of perchloric acid (PCA, final

concentration approximately 1.1%). Samples were then frozen at -70°C until analysis. After thawing, samples were centrifuged (Beckman Coulter Microfuge® R) at 16,000 g for 5 min; supernatants were neutralized with 1 M K_2CO_3 and recentrifuged. cAMP was determined in supernatants using the EIA procedure noted above.

2.2.9 Plasma measurements

In the chasing experiments, plasma samples were analyzed for catecholamines, cortisol, glucose and lactate. The plasma catecholamines, ADR and NADR were measured as described by Perry *et al.* (1996). Plasma cortisol was measured with a commercial radioimmunoassay (RIA) kit (ICN) as described in Dugan and Moon (1998). Both plasma glucose (Moon *et al.*, 1999) and plasma lactate (Bergmeyer, 1983) were measured enzymatically. For the agonist feeding experiments, plasma CLEN was measured qualitatively using the commercial EIA kit available from Neogen Corp. (Lexington, KY).

2.2.10 Statistical Analysis

Statistical differences were evaluated using appropriate tests depending upon the experiment (Sigma Stat version 2.0, SPSS Inc.). These included 1-way ANOVA, 1-way repeated measures ANOVA, 2-way ANOVA, or 2-way repeated measures ANOVA followed by an appropriate multiple comparison test. Level of significance was taken to be $P<0.05$.

2.3 Results

2.3.1 Pharmacological classification

Competitive displacement assays using selective and non-selective agonists and antagonists were used to verify the presence of a β_2-AR in rainbow trout hepatic tissue. The agonists and antagonists that were selective or had higher affinity for the β_2-AR displaced the non-selective ^{3}H-CGP more efficiently than the β_1-AR ligands (Fig. 2.1). Inhibitory constants (K_i) for the ligands are presented in Table 2.1.

2.3.2 Physical stress

A significant decrease ($P<0.05$) in the number of ^{3}H-CGP binding sites (B_{max}) was seen only after 1 d of chasing compared with non-chased controls; no significant change in receptor affinity (K_d) for any sample period was observed (Fig. 2.2). Plasma values for catecholamines, cortisol, glucose and lactate are presented in Table 2.2. Chasing significantly increased plasma ADR only after 1 d ($P<0.05$). Plasma catecholamine levels are elevated in part due to the sampling method, however the handling of fish was identical for both groups. Significant increases in plasma cortisol and lactate occurred at all three sampling periods ($P<0.05$). No significant changes were noted in either plasma NADR or plasma glucose. High between-sample variation may have masked other significant changes.

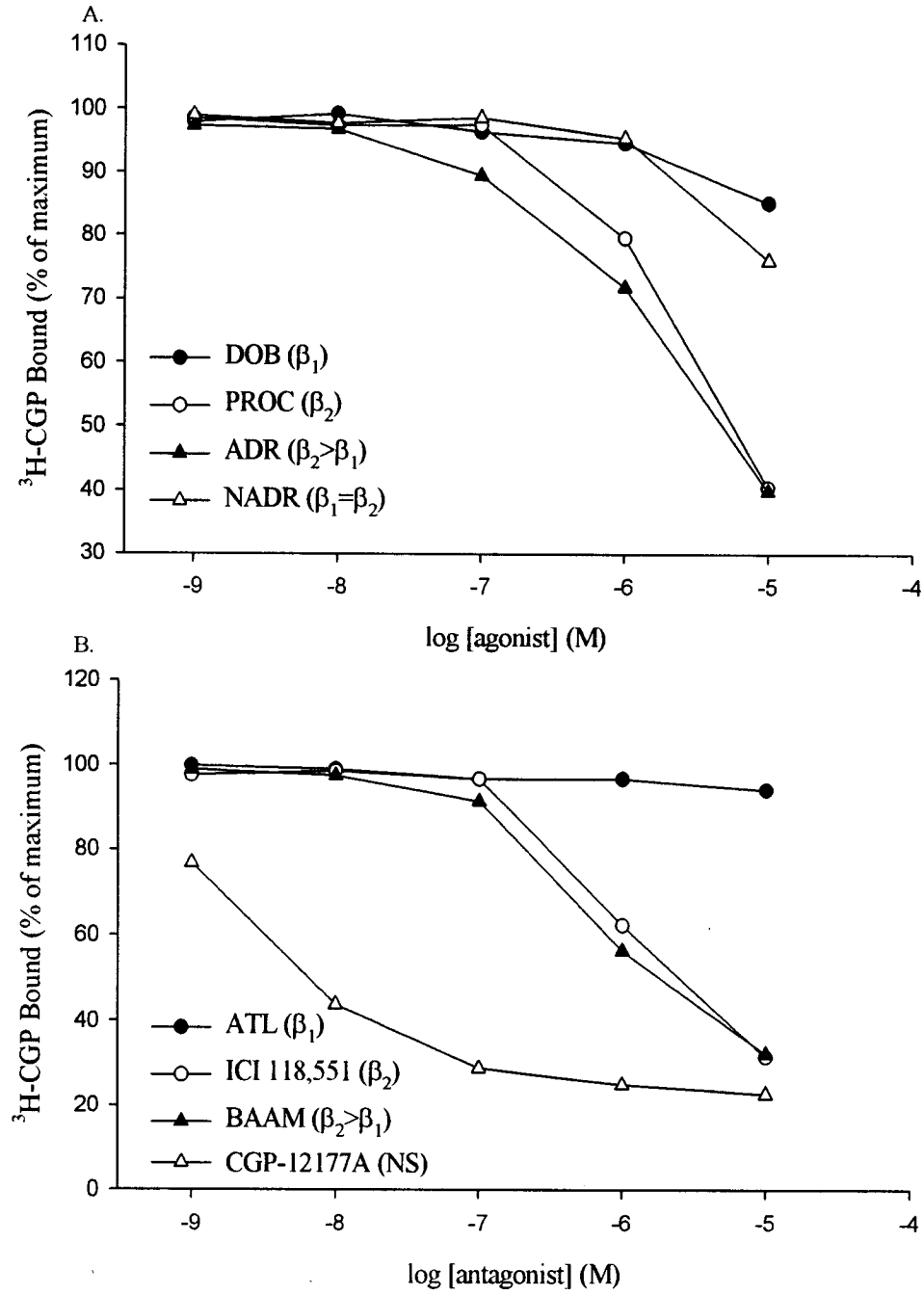


Figure 2.1. Competitive displacement of ^{3}H-CGP-12177 binding to rainbow trout hepatic membranes by various agonists (A.) and antagonists (B.). Semi-purified membranes were incubated for 1 h with a saturating concentration of ^{3}H-CGP (1 nM) with increasing concentrations of (A.) dobutamine (DOB), procaterol (PROC), adrenaline (ADR) or noradrenaline (NADR); and (B.) atenolol (ATL), ICI 118,551, bromoacetyl alprenolol menthane (BAAM), or CGP-12177A. Values are means (n = 4-5). SEM for each data point was less 5% and is omitted for clarity. Data modified from Nickerson *et al.* (2001). Subtype selectivity of the ligands is shown in parentheses; NS represents non-selective.

Table 2.1. Inhibitory constants (K_i) for ^{3}H-CGP-12177 binding in rainbow trout hepatic membranes. Values are means ± SEM (n = 4-5). Ligands where 50% inhibition was not achieved at the highest concentration (10 μM) are represented by N/A. Current mammalian pharmacological classification for the ligands is presented in parentheses.

AGONIST	K_i (M)
Dobutamine (β_1)	N/A
Procaterol (β_2)	$3.3 \pm 0.6 \times 10^{-6}$
Adrenaline ($\beta_2 > \beta_1$)	$3.0 \pm 1.0 \times 10^{-6}$
Noradrenaline ($\beta_1 = \beta_2$)	N/A
ANTAGONIST	
Atenolol (β_1)	N/A
ICI 118,551 (β_2)	$1.3 \pm 0.5 \times 10^{-6}$
BAAM[a] ($\beta_2 > \beta_1$)	$7.1 \pm 0.9 \times 10^{-7}$
CGP-12177A (non-selective)	$4.3 \pm 1.7 \times 10^{-9}$

[a] bromoacetyl alprenolol menthane

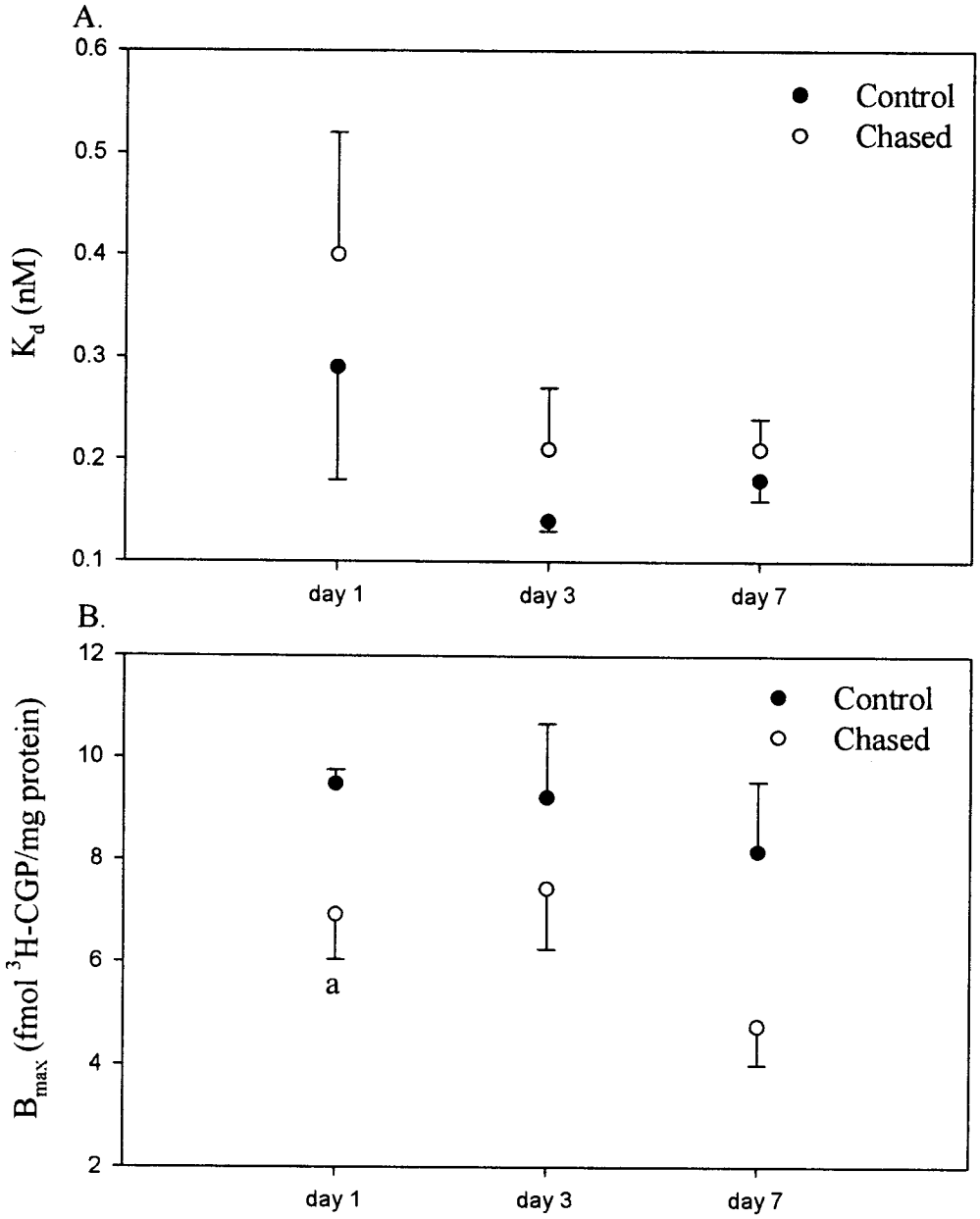


Figure 2.2. Effect of chasing rainbow trout for 1,3, or 7 d on hepatic β_2-AR affinity (A.) and number of CGP-binding sites (B). Approximately 220-265 μg of membrane protein were incubated for incubated for 1 h at room temperature with increasing concentrations (0.1-5.0 nM) of ^{3}H-CGP alone (total binding) or with 10 μM alprenolol (ALP; non-specific binding). K_d and B_{max} values were calculated using EBDA (Munson and Rodbard, 1980). Values represent means ± SEM (n = 5-6). Significant difference from control is indicated by the letter **a** (2-way ANOVA followed by Tukey test, $P < 0.05$). No significant effect of time or treatment x time interaction was observed.

Table 2.2. Plasma values for chased and control rainbow trout. Blood was sampled by caudal puncture 1 h post chasing. Values are means ± SEM (n = 5-6). Significant difference from control indicated by the letter **a** (2-way ANOVA followed by Tukey test, $P<0.05$).

PLASMA	TREATMENT	1 day	3 day	7 day
Adrenaline (nM)	Chased	435.3 ± 65.6[a]	182.6 ± 57.5	90.9 ± 27.2
	Control	87.0 ± 33.5	99.2 ± 41.0	47.1 ± 14.8
Noradrenaline (nM)	Chased	205.3 ± 71.6	83.2 ± 23.6	56.8 ± 11.2
	Control	57.6 ± 21.9	60.4 ± 18.6	46.2 ± 10.8
Cortisol (ng/ml)	Chased	125.9 ± 27.8[a]	137.1 ± 28.2[a]	119.3 ± 30.6[a]
	Control	5.63 ± 3.15	8.28 ± 5.19	1.50 ± 0
Glucose (mM)	Chased	6.60 ± 1.21	7.78 ± 1.02	6.27 ± 1.85
	Control	3.81 ± 0.45	5.52 ± 1.59	3.82 ± 0.85
Lactate (mM)	Chased	7.03 ± 0.73[a]	4.43 ± 0.75[a]	4.35 ± 0.41[a]
	Control	0.73 ± 0.07	0.76 ± 0.03	0.90 ± 0.06

2.3.3 Agonist feeding

After 37 d of feeding food containing CLEN or RACT, a significant decrease ($P<0.001$) in B_{max} was seen only in the CLEN fed fish; no change in K_d was seen for either agonist (Fig. 2.3). Although statistically not significant, a strong trend towards a decrease in β_2-AR mRNA levels was observed in trout fed CLEN (Fig. 2.4). Plasma CLEN concentration in 5 of the 8 CLEN-fed fish was above the detection limit of the kit (>31.4 nM), with a minimum mean level of 21.3 ± 4.9 nM (n = 8); CLEN levels in SHAM-fed trout were below kit detection (<0.1 nM). No commercial method for determining plasma RACT values is currently available. While a significant decrease in the number of binding sites was observed in the CLEN-fed fish, no significant effect was observed in ACase activities between CLEN and SHAM-fed fish (Fig. 2.5). Only 10 μM FSK significantly increased ACase activities in both groups of fish above their respective controls ($P<0.05$). Displacement studies showed the trout hepatic β_2-AR has a higher affinity for CLEN than RACT with mean K_i values of 4.34×10^{-7} M and 70.9×10^{-7} M, respectively (Fig. 2.6). To determine if CLEN and RACT act as agonists on the trout hepatic β_2-AR function, cAMP production was measured in isolated hepatocytes from rainbow trout fed a normal (control) diet. Both 10 nM and 100 nM CLEN and RACT failed to stimulate cAMP production above basal levels (data not shown). However, both CLEN and RACT at 10 μM significantly elevated cAMP levels by 2.2- and 4.6-fold, respectively, above the 15 min control (15'CTL). Conversely, 10 μM CLEN was more effective than 10 μM RACT at inhibiting 100 nM ISO-stimulated cAMP production. CLEN blocked ISO-stimulated cAMP production to the level of the 15'CTL while RACT inhibited ISO-stimulated production by approximately 65%. When tested as an ISO-antagonist, a 10 min pre-incubation with CLEN and RACT was undertaken prior to the

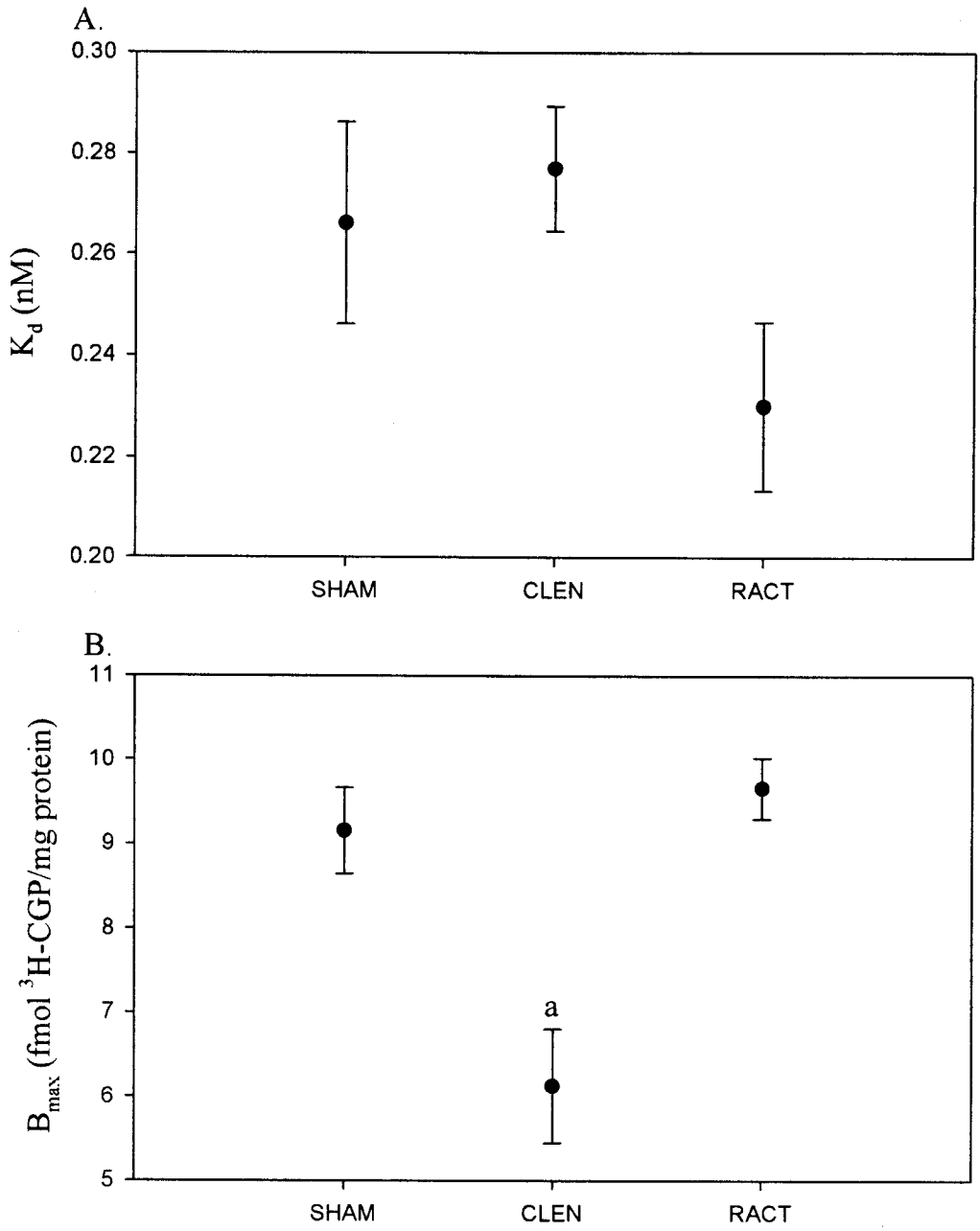


Figure 2.3. Effect of feeding the β_2-agonists clenbuterol (CLEN) and ractopamine (RACT) on rainbow trout hepatic β_2-AR affinity (A.) and number of CGP-binding sites (B.). Values of K_d and B_{max} calculated as in Fig. 1. Values represent means ± SEM (n = 7-8). Significant difference from SHAM fed fish is indicated by the letter **a** (1-way ANOVA followed by Tukey test, $P<0.001$).

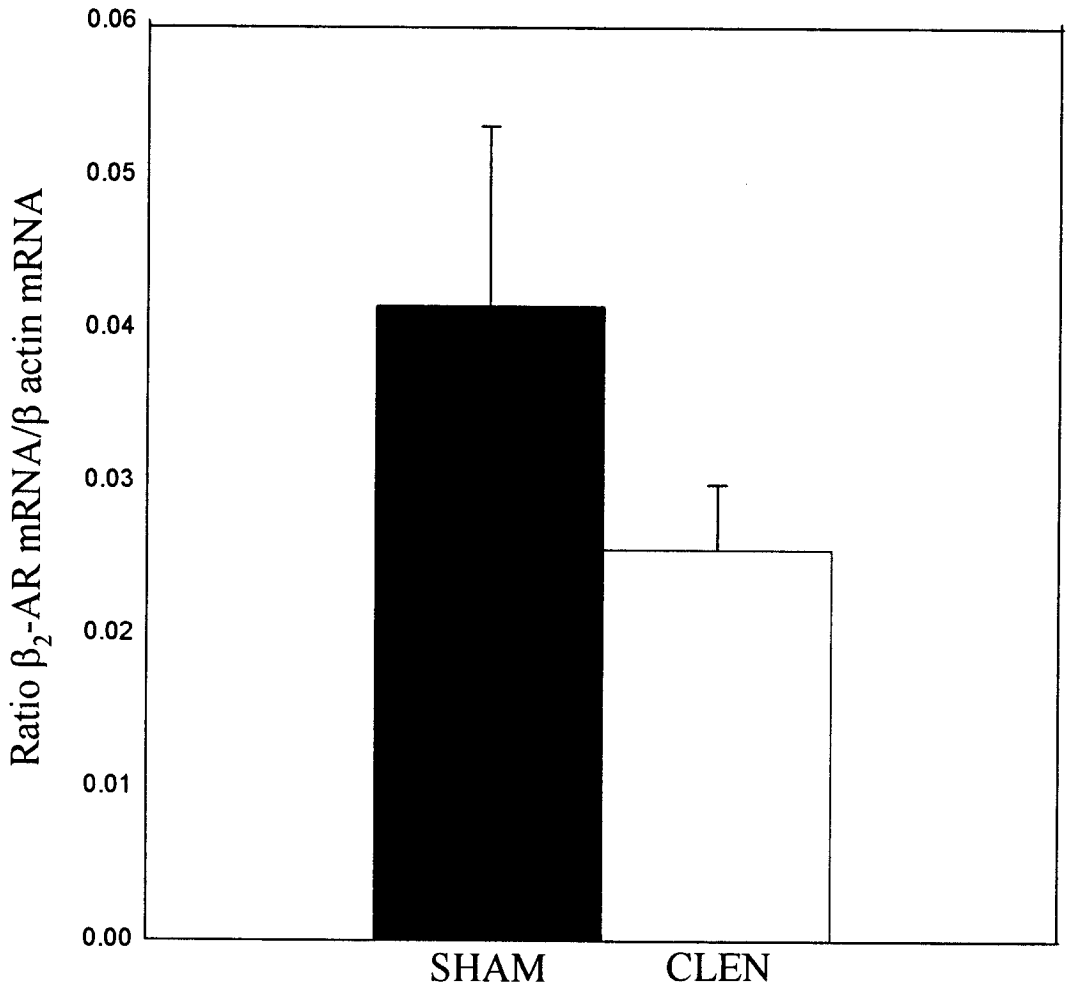


Figure 2.4. Rainbow trout hepatic β_2-AR mRNA levels relative to β-actin in SHAM (black bar) and CLEN (white bar) fed fish. Bars represent means ± SEM (n = 6). No significant difference was observed (Mann-Whitney rank sum test, $P>0.05$).

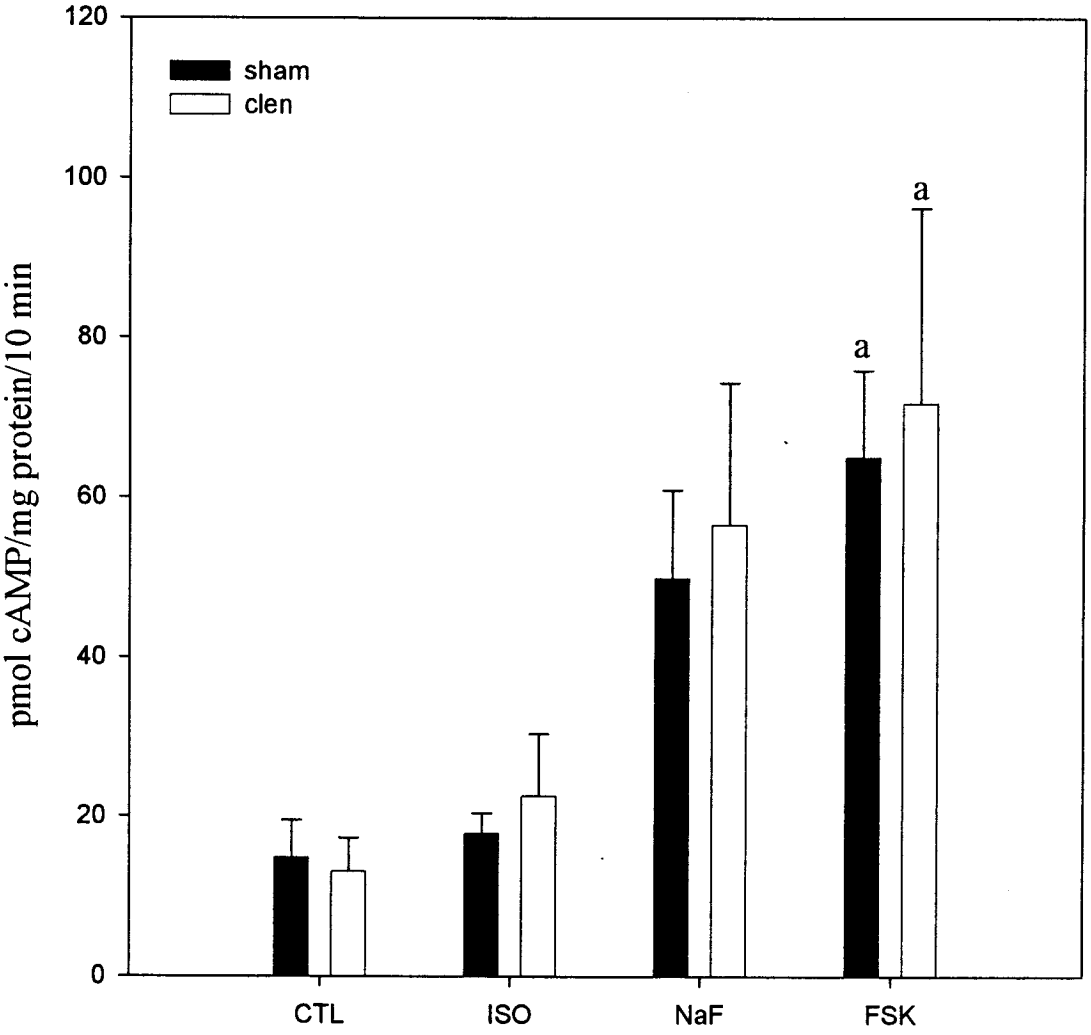


Figure 2.5. Hepatic adenylyl cyclase (ACase) activity in SHAM and CLEN-fed rainbow trout. Membranes were incubated with either saline (CTL), 100 μM isoproterenol (ISO), 10 mM NaF, or 10 μM forskolin (FSK). Bars represent means + SEM (n = 3). Significant difference from respective controls is indicated by the letter **a** (2-way repeated measures ANOVA followed by Tukey test, $P<0.05$).

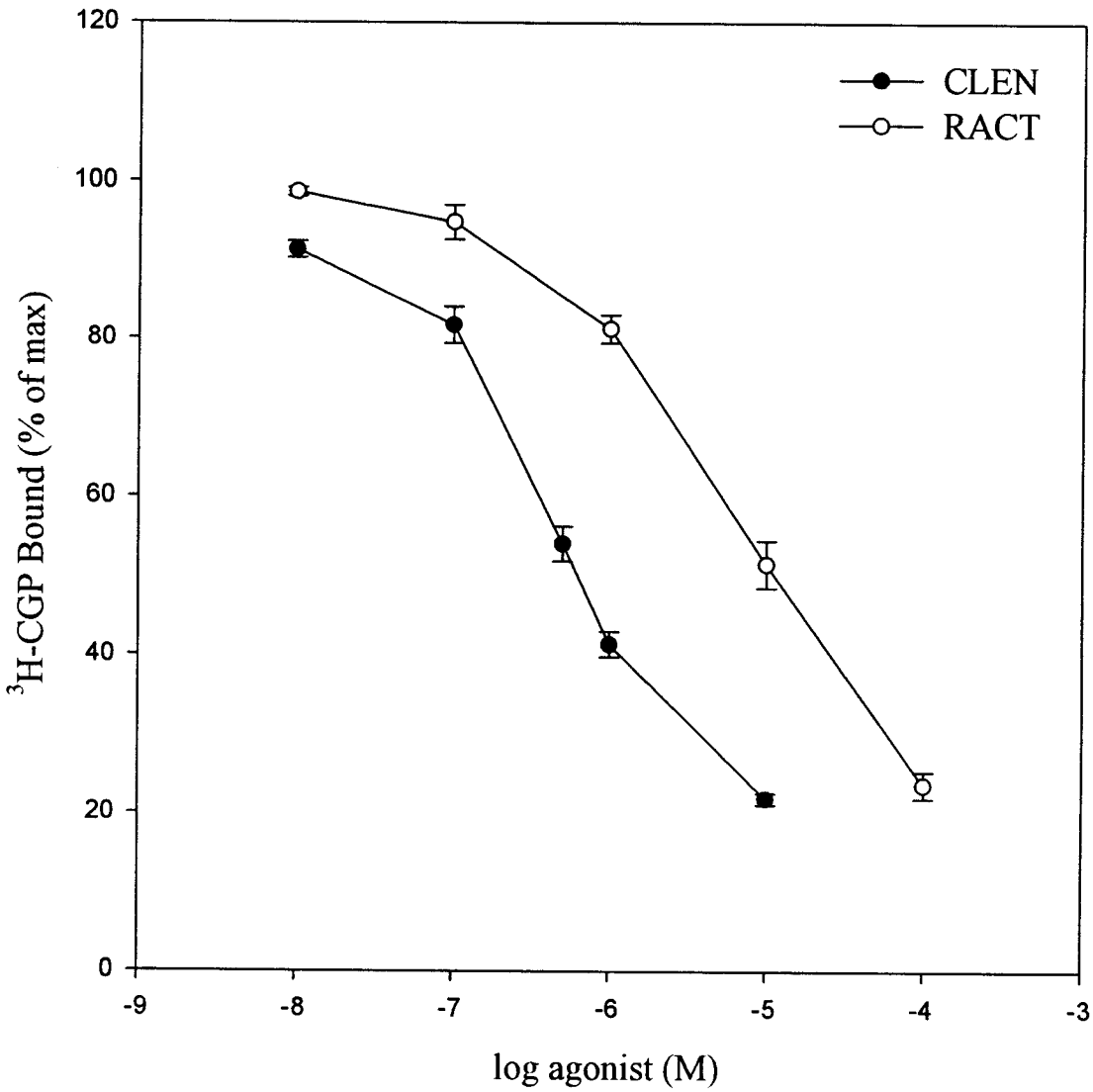


Figure 2.6. Displacement of ^{3}H-CGP-12177 by the β_2-agonists clenbuterol (CLEN) and ractopamine (RACT) in rainbow trout semi-purified hepatic membranes. Approximately 280 μg of membrane protein were incubated for 1 h with a saturating concentration of ^{3}H-CGP (1 nM) with increasing concentrations of either CLEN or RACT. Values represent means ± SEM (n = 5-6). CLEN and RACT K_i values were 4.34×10^{-7} M and 7.09×10^{-6} M, respectively, calculated using EBDA (Munson and Rodbard, 1980).

addition of ISO at time 0', while CLEN and RACT preincubation controls were handled in an identical fashion but received saline rather than ISO at time 0'. On a per minute basis, no difference in cAMP production as observed between the RACT control and 10 μM RACT, and each value was approximately 4-fold above the 15'CTL. Conversely, the CLEN control was similar to level of the 15'CTL and was significantly lower than 10 μM CLEN (Fig. 2.7).

2.4 Discussion

This study examined the effects of a physical stressor (chasing to exhaustion), and prolonged agonist feeding on the characteristics of the hepatic β_2-AR of rainbow trout. The ability of the β_2-AR selective ligands to displace ^{3}H-CGP more effectively than the β_1-AR selective ligands supports the presence of a β_2-AR in hepatic tissue of rainbow trout (Fig. 2.1), which is in agreement with a previous pharmacological classification done in isolated hepatocytes (Reid *et al.*, 1992). This is also consistent with β_2-ARs being the predominant β-AR subtype found in the liver of other vertebrate species including the European eel, *Anguilla anguilla* (Fabbri *et al.*, 2001), channel catfish, *Ictalurus punctatus* (Finkenbine *et al.*, 2002), Australian lungfish, *Neoceratodus forsteri*, the axolotl, *Ambystoma mexicanum*, the toad, *Xenopus laevis* (Janssens and Grigg, 1988), and the dog, *Canis familiaris* (García-Sàinz *et al.*, 1996). A single class of ^{3}H-CGP binding sites in purified hepatic membranes from rainbow trout was shown by Scatchard analyses (Fabbri *et al.*, 1995a; Dugan and Moon, 1998).

Daily chasing to exhaustion for 7 d resulted in a significant 27% decrease of ^{3}H-CGP binding sites after 1 d (Fig. 2.2B). Perry *et al.* (1996) found a 20% reduction in the number of red blood cell β-ARs in rainbow trout on day 7 of chasing, the only time period the authors examined binding parameters. The lack of a significant decrease in the other

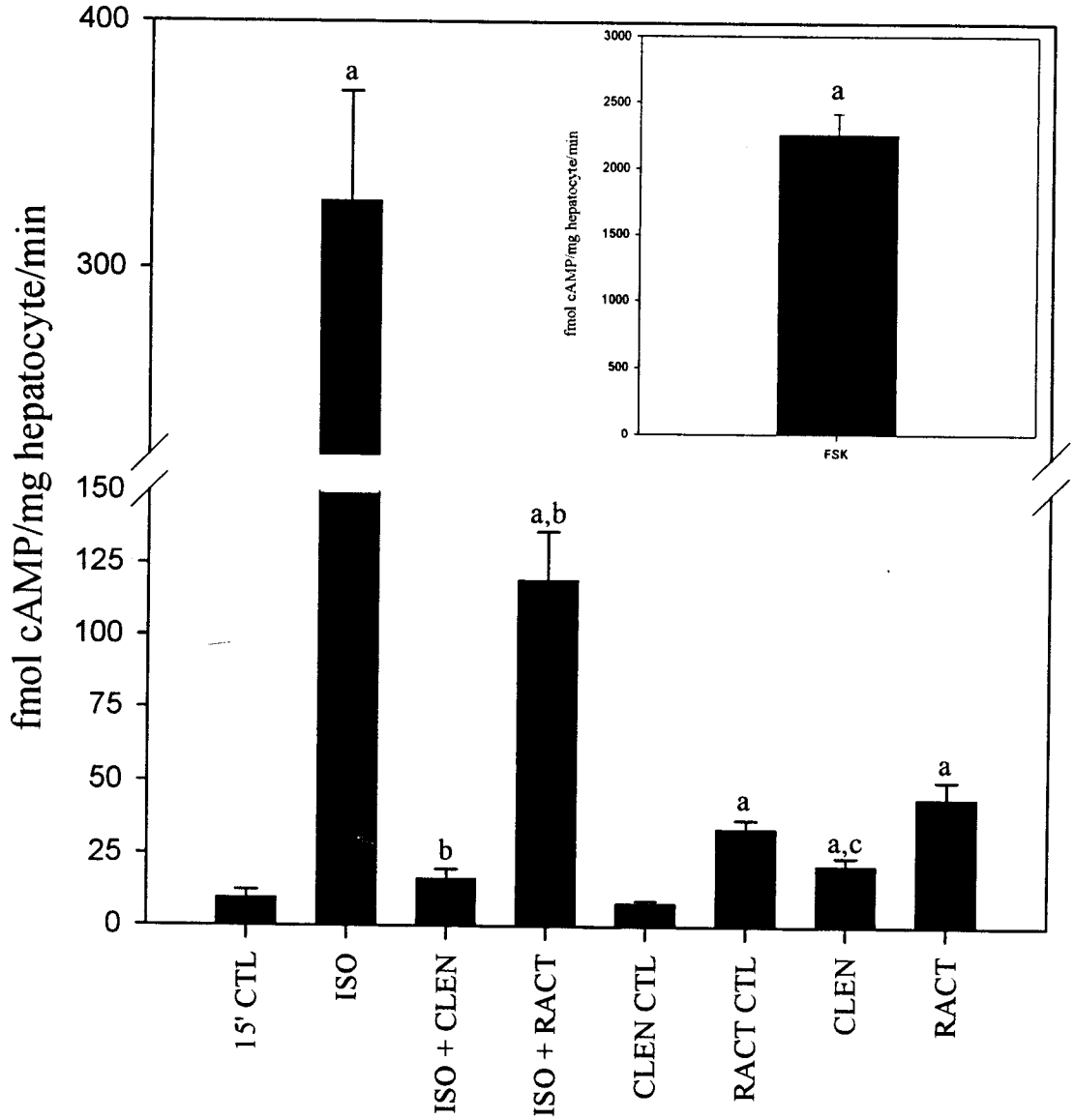


Figure 2.7. cAMP production in isolated rainbow trout hepatocytes. Hepatocytes were incubated with either saline (15'CTL), 100 nM isoproterenol (ISO) alone or in combination with either 10 μM clenbuterol (CLEN) or ractopamine (RACT), 10 μM CLEN or RACT alone or 10 μM forskolin (FSK; inset). CLEN CTL and RACT CTL represent hepatocytes that were preincubated with CLEN or RAC but received saline instead of ISO at time zero. Bars represent means + SEM (n = 6). Significant difference from saline control (15'CTL) is indicated by the letter **a**; form ISO by the letter **b**; from clenbuterol control (CLEN CTL) by the letter **c** (1-way repeated measures ANOVA followed by Tukey test, $P<0.05$).

sampling periods reported here may be related to a decrease in catecholamine release. Plasma ADR was significantly elevated above control levels only after 1 d and the amount released was significantly decreased after daily chasing for 7 d (Table 2.2). Reid *et al.* (1994) showed that 5 d of exercise to exhaustion decreased the responsiveness of chromaffin cells to agonist-stimulated catecholamine release. In addition, plasma cortisol was significantly elevated for all 3 sampling periods (Table 2.2). Elevating plasma cortisol using mini-osmotic pumps to approximately 130 ng/ml for 10 days was previously shown by Reid *et al.* (1992) to up-regulate the number of cell surface β_2-ARs in isolated rainbow trout hepatocytes. However, no up-regulation was observed in our experiment; instead there was a strong trend towards a decrease in the number of binding sites at 7 d of chasing (Fig. 2.2B). As well, no effect of elevated plasma cortisol on the number of β_2-ARs in purified hepatic membranes from rainbow trout has been shown (Dugan and Moon, 1998). These contrasting observations of the impact of cortisol on hepatic β_2-ARs may reflect the different methods used in the two studies (hepatocytes versus hepatic membranes).

The impact of prolonged feeding of CLEN and RACT twice daily for 37 d on trout hepatic β_2-ARs was also determined. Similar to the physical stress study, a decrease of 33% in the number of ^{3}H-CGP binding sites was observed in the CLEN- but not RACT-fed trout (Fig. 2.3B). While minimal mean plasma CLEN levels were found to be only 21 nM, trout fed CLEN have been found to accumulate the agonist in the liver. Brambilla *et al.* (1994) found rainbow trout fed 5 ppm CLEN for 21 d achieved the highest mean levels of 440 ppb CLEN in the liver after 15 d of treatment and CLEN was detectable 30 d after agonist feeding had ceased. CLEN was shown to cause down-regulation in mammals of up to 75%

in rat cerebellum β_2-ARs with no change in K_d after eight daily i.p. injections (Ordway *et al.*, 1987; Vos *et al.*, 1987).

The lack of a change in the number of binding sites in the RACT fed fish may be related to the stereoisomer composition of the RACT used in the study. The RR and RS stereoisomers have the highest affinity for porcine β_2-ARs (Mills *et al.*, 2003b) with RR being the most biologically active (Mills *et al.*, 2003a). These two stereoisomers combined only composed approximately 50% of the RACT used in this experiment, which most likely limited the effectiveness of RACT to alter the hepatic β_2-AR binding parameters.

The ability of prolonged agonist exposure to down-regulate β-ARs in rainbow trout has been shown to vary with subtype, tissue and potentially with the method used. Gilmour *et al.* (1994) found a 33% decrease in rainbow trout red blood cell β-AR receptor numbers after 3 d of elevated plasma catecholamines using mini-osmotic pumps. In contrast, Gamperl *et al.* (1994b) found no change in rainbow trout cardiac β-ARs with five injections of catecholamines over a 6 h period. Although a trend towards an increase was seen, no significant change in B_{max} was found in either red or white muscle membranes in trout fed CLEN for 37 d, while a significant increase in K_d in both muscle types did occur (Lortie *et al.*, 2004). Neither Gilmour *et al.* (1994) nor Gamperl *et al.* (1994b) reported a change in K_d after prolonged exposure to agonists.

Although there was a highly significant decrease in the number of hepatic CGP-binding sites in the CLEN-fed trout, no significant effect was observed on activation of ACase (Fig. 2.5). Auman *et al.* (2001a,b) showed a decrease in the number of hepatic β_2-AR with daily injections of the β_2-agonist terbutaline with no change in ACase activity in neonatal rat livers. In fact, these authors found a heterologous sensitization despite decreases

in receptor number. In the CLEN-fed trout, a slight trend towards sensitization was observed when compared to the SHAM fed trout. The lack of a change in ACase activity despite a decrease in the number of binding sites may be due to the presence of spare receptors. Brown *et al.* (1992) reported 50% of maximal response with 1 to 25% receptor occupancy in human heart, depending on the region. Thus, the 33% decrease observed in this study may not be sufficient to alter ACase activity.

The decrease in the number of CGP-binding sites was associated with corresponding strong trend in decreasing hepatic β_2-AR mRNA levels in CLEN-fed trout (Fig. 2.4). Agonist-induced decreases in β-AR mRNA levels with corresponding decreases in receptor number after exposure to ISO have been reported in S49 mouse lymphona cells (Hadcock *et al.*, 1989a) and DDT_1 MF-2 hamster cells (Hadcock and Malbon, 1988; Hadcock *et al.*, 1989b).

While competition studies show the rainbow trout hepatic β_2-AR has a higher affinity for CLEN than RACT (Fig. 2.6), RACT was more effective in stimulating cAMP production in isolated hepatocytes (Fig. 2.7). In fact, for rainbow trout hepatic β_2-ARs, CLEN appears to be only a weak agonist and tends to act more as an antagonist by successfully blocking ISO-stimulated cAMP production (Fig. 2.7). CLEN acted only as a partial agonist in mammals with respect to cAMP production. The maximum cAMP production by CLEN was only 23% that of ISO in female rat skeletal muscle (Sillence *et al.*, 1995). Ordway *et al.* (1987) reported similar results in rat cerebellum. However, in addition to decreasing the number of binding sites, CLEN may also cause hepatic β_2-AR desensitization. On a per min basis, the CLEN CTL that had a total of 25 min of 10 μM CLEN exposure due to the 10 min preincubation, had significantly lower cAMP production than the hepatocytes that had the 15

min of 10 μM CLEN exposure (Fig. 2.7). Conversely, no desensitization was observed in hepatocytes incubated with RACT. In C_2C_{12} mouse skeletal muscle cell cultures, 10 μM RACT to increased cAMP production 7.2- and 6.9-fold in myoblasts and myotubes, respectively, (Shapell *et al.*, 2000) values comparable with the 4.6-fold increase observed in this study.

Both physical stress and prolonged exposure to CLEN and RACT significantly decreased the number of hepatic β_2-AR binding sites in rainbow trout. To my knowledge, this is the first report of a decrease in the number of β_2-AR binding sites in any rainbow trout tissue. No significant difference in the number of myocardial β-AR binding sites was found in rainbow trout exposed to moderate hypoxia for 6 h (Gamperl *et al.*, 1998). However, the trout heart β-AR subtype composition is complex with expression of both the β_2-AR (Nickerson *et al.*, 2001) and β_{3a} (Nickerson *et al.*, 2003) mRNA reported, but which functions as the predominant subtype is not known at this time. The mechanisms of down-regulation and desensitization of the β_2-AR in the mammalian system has been linked to phosphorylation of the receptor at key sites in the G-protein binding domain (intracellular domain 3) and the cytoplasmic tail (Lohse, 1993; Barnes, 1995). However, sequencing of the rainbow trout β_2-AR found the corresponding phosphorylation sites in the G-protein binding domain are lacking and relatively few potential phosphorylation sites were found in the tail (Nickerson *et al.*, 2001). The possible role these phosphorylation sites may have in the decreasing binding site numbers is currently unclear. Another mechanism not involving receptor phosphorylation may be involved in the loss of the observed binding sites. Non-phosphorylation mediated down-regulation of a mutant β_2-AR lacking carboxy-terminal phosphorylation sites was reported in Chinese hamster fibroblast CHW cells (Bouvier *et al.*,

1988). mRNA from both β-arrestin and βARK (β-adrenergic receptor kinase) have been detected in trout liver but to a lesser extent than in red blood cells (Nickerson *et al.*, 2004). In mammals, no detectable expression of β-ARK mRNA and relatively low levels of β-arrestin mRNA in hepatic tissue have been observed (Benovic *et al.*, 1989; Lohse *et al.*, 1990) and down-regulation and desensitization of hepatic β_2-AR by 50% has been shown (Medina-Martínez and García-Sáinz, 1993). The function of β-arrestin and β-ARK in trout hepatic tissue and any potential role in the agonist regulation of ^{3}H-CGP binding sites requires further attention.

Chapter 3: Rainbow trout gill β-adrenoceptors

3.1 Introduction

Apart from gas exchange, the gills of fish have other important physiological roles including ionic and acid-base regulation, nitrogen excretion and hormone metabolism (Morgan and Potts, 1995; Olson, 2002). All of these processes are performed by the cells forming the gill epithelium, which consist of two predominant cell types, pavement or principal cells (PVC) and mitochondria-rich (MR) or chloride cells. Over 90% of the gill epithelium is occupied by the PVC and are primarily responsible for gas transfer while the MR cells are primarily responsible for ionic regulation (Perry, 1998; Wilson and Laurent, 2002). However, PVCs are also known to play a role in ionic regulation, although to a lesser extent than the MR cells (Galvez *et al.*, 2002; Marshall, 2002).

An adrenergic role in ionic regulation in fish gills is well established. Mendelsohn *et al.* (1981) showed Cl^- secretion in killifish (*Fundulus heteroclitus*) gills as a result of isoproterenol (ISO)-stimulated cAMP production. Similarly, Perry *et al.* (1984) showed catecholamine (CA)-induced changes in Cl^- flux in rainbow trout (*Oncorhynchus mykiss*) gills. In addition, ISO-induced changes in intracellular ion concentrations within PVC and MR cells in brown trout (*Salmo trutta*) is reported (Morgan and Potts, 1995).

β-Adrenoceptors (β-AR) are known to be present in the gills of fish (see Nekvasil and Olson, 1985 for references). However, previous adrenergic studies in gills have focused primarily on vascular ARs and their role in regulating gill blood flow (Payan and Girard, 1977; Sundin, 1995). Recent molecular evidence supports the existence of a β_{3a}- and to a lesser extent a β_2-AR in rainbow trout gill tissue (Nickerson *et al.*, 2001, 2003). To date, no

studies have attempted to pharmacologically characterize the β-ARs in gills. Thus, the purpose of this study was to pharmacologically characterize the β-ARs in the rainbow trout gill and to examine the impact of exposure to hypercarbia on gill β-AR binding kinetics. Hypercarbia is an environmental stressor that significantly elevates plasma CAs (McKendry and Perry, 2001; Perry and Reid, 2002).

3.2 Materials and Methods

3.2.1 Animals

Female rainbow trout weighing approximately 150–600 g were obtained from Linwood Acres Trout Farm (Campbellcroft, ON) and transported to the University of Ottawa. Fish were held under a 12h light /12h dark photoperiod in 1250 l tanks in dechloraminated city of Ottawa tap water at 13°C (±1°C). The fish were fed trout pellets (Purina Trout Chow) 5 times a week. Fish were allowed to acclimate for a minimum of 4 weeks before use. Experiments were conducted between January to March (characterization) and December to January (hypercarbia) and commenced between the hours of 10 am and 2 pm. Animals were cared for and treated in accordance with guidelines from the Canadian Council on Animal Care.

3.2.2 Membrane preparation

Crude plasma membranes were prepared from gill cells using a modification of the method of Guibbolini and Lahlou (1987). Fish weighing approximately 400-600g were randomly sampled from the holding tank, killed by a quick blow to the head and a ventral incision made to expose the heart. The bulbous arteriosus was cannulated and the gills were

perfused with 0.9% NaCl to clear the blood. Gill arches were then removed and the gill cells scraped from the filaments with the blunt side of a razor blade. The cells were resuspended in 0.3 M sucrose (pH 7.6) and homogenized with a Wheaton stirrer. This homogenate was then filtered through a nylon filter (pore size 250 μm) and centrifuged (Sorvall RC-5B Plus) at 1,000 g for 10 min at 4°C. The resulting supernatant was then centrifuged at 25,000 g for 10 min at 4°C. The pellet was then resuspended in a small volume of 0.3 M sucrose and frozen at -20°C for future radioactive binding studies.

3.2.3 Hypercarbia exposure

Eight trout per exposure weighing 150-200g were placed in two separate black acrylic boxes with flowing, aerated water and allowed to acclimate overnight. Fish in each box were kept in individual compartments separated by opaque dividers. One box of four fish received water made hypercarbic by gassing a water equilibration column with 1.5% CO_2 in air (Cameron flowmeter model GF-3/MP) for a target water P_{CO_2} of 8 mm Hg (P_{O_2} of 155 mm Hg) as described in Perry and Reid (2002). The other box containing four control fish in individual compartments continued to receive normocarbic, aerated water (P_{CO_2} of 0.3 and P_{O_2} of 155 mm Hg). This treatment continued for 2 d at which time fish were killed by a blow to the head and the gills quickly excised for cell isolation.

3.2.4 Gill cell preparation

Gill cells (mixed PVC and MR) were isolated using a modification of the method of Galvez *et al.* (2002). Gill arches were excised and rinsed in Hanks' saline (in mM: 136.7 NaCl, 5.4 KCl, 0.8 $MgSO_4$, 0.33 NaH_2PO_4, 0.44 KH_2PO_4, 5.0 Hepes, 5.0 Hepes-Na, and 1.0

$NaHCO_3$, pH 7.6) to eliminate excess blood and mucous. Filaments were removed from the gill rakers and cut into small sections. The cut filaments were then placed in 5 ml trypsin-EDTA (Gibco) and shaken for 8 min at 300 rpm on a gyrotory shaker. Trypsinization was halted by passing the digest through a 100 µm nylon cell strainer into a centrifuge tube containing 20 ml Hanks' saline with 10% fetal bovine serum (FBS). The tissue remaining on the filter was recovered and the trypsinization and filtration protocol was repeated twice more with the digest being collected into the same stop buffer. The final filtrate was then centrifuged at 300 g for 8 min at 4°C (Sorvall RC 5B Plus). The resulting pellet was resuspended in a small volume of double distilled water for a maximum of 1 min to lyse any remaining red blood cells. This incubation was terminated by filling the centrifuge tube with Hanks' saline and centrifuging as before. The pellet was resuspended in Hanks' saline, filtered through a 100 µm nylon cell strainer to remove any cellular debris and recentrifuged. The final pellet was resuspended in a small volume of Hanks saline at a concentration of approximately 5-20 mg cells per ml. Attempts to separate PVC from MR cells according to the method of Galvez *et al.* (2002) could not positively separate two distinct cell populations and therefore binding studies were done on the total gill cell population. Cells were counted using a hemacytometer (Spotlite^{TM}, American Scientific Products) and viability was verified by trypan blue exclusion and was at least 65% for binding experiments.

3.2.5 Binding studies

Protein concentration of the membrane preparations was determined using the bicinchoninic acid (BCA) protein assay (Sigma) with bovine serum albumin (BSA) as standard. Gill cell and membrane β-ARs binding kinetics (K_d, B_{max}) were determined using

the hydrophilic β-antagonist ((-)-4-(3-t-butylamino-2-hydroxypropoxy)-[5,7-^{3}H]benzimidazol-2-one) ^{3}H-CGP-12177 (^{3}H-CGP; Amersham, specific activity 52.0 Ci/mmol) as described in Chapter 2. For membrane binding studies, 75-100 μg membrane protein per 50 μl was used and all incubations were performed in 0.3 M sucrose. Competition studies to characterize the gill β-AR in crude membranes used a saturating concentration of 2 nM ^{3}H-CGP alone or with increasing concentrations (1 nM to 10 μM) of selective and non-selective agonists and antagonists as described in Chapter 2. All binding assays were terminated by 4 washes of ice cold 0.9% NaCl with a cell membrane harvester (Brandel 24R). The incubations were collected onto borosilicate filters (#32 glass fibre filters, Schleicher and Schuell) and placed in polyethylene scintillation vials containing 4 ml scintillation cocktail (ACS II; Amersham or Ready-Safe^{TM}; Beckman Coulter). Vials were left in the dark at room temperature for at least 24 h before radioactivity was determined using a Beckman Coulter LS 6500 liquid scintillation counter. Binding parameters (K_d, K_i, and B_{max}) were determined using the EBDA and LIGAND computer programs (Munson and Rodbard, 1980).

3.2.6 Statistics

Statistical differences in binding parameters were evaluated using 1-way ANOVA (Sigma Stat version 2.0, Jandel Scientific). Level of significance was taken to be $P<0.05$.

3.3 Results

3.3.1 Characterization

Specific binding of ^{3}H-CGP to semi-purified gill membranes was saturable as shown in Fig. 3.1. Scatchard analysis was linear supporting a one-site model with a high affinity for ^{3}H-CGP (data not shown). Binding kinetics in the membrane preparations produced a mean K_d of 0.30 ± 0.11 nM and a B_{max} of 33.2 ± 6.75 fmol/mg protein (± SEM, n = 5).

Competition studies using selective and non-selective agonists and antagonists were done to pharmacologically characterize the gill β-AR (Fig. 3.2). For the non-selective agonists, ISO was the most effective with NADR and ADR displacing ^{3}H-CGP with almost equal effectiveness. The β_1-AR agonist dobutamine (DOB) was the most effective of the selective agonists at displacing the non-selective ^{3}H-CGP. However, the β_1-AR selective antagonist atenolol (ATL) was ineffective as 10 μM failed to inhibit more than 50% of ^{3}H-CGP binding. The non-selective CGP-12177A and propranolol (PROP) were the most effective of all the ligands while the β_3-AR antagonist SR 59230A was the most effective of the selective antagonists. Inhibitory constants (K_i) for the agonists and antagonists are shown in Table 3.1.

3.3.2 Hypercarbia exposure

Preliminary binding studies on isolated gill cells from trout randomly sampled from the main holding tank found a mean affinity (K_d) of 0.82 ± 0.09 nM and 2015 ± 113 binding sites per cell (B_{max}) (± SEM, n = 3). After exposing rainbow trout to hypercarbia for 2 d, no significant difference was observed in isolated gill cells for either K_d or in B_{max} compared to

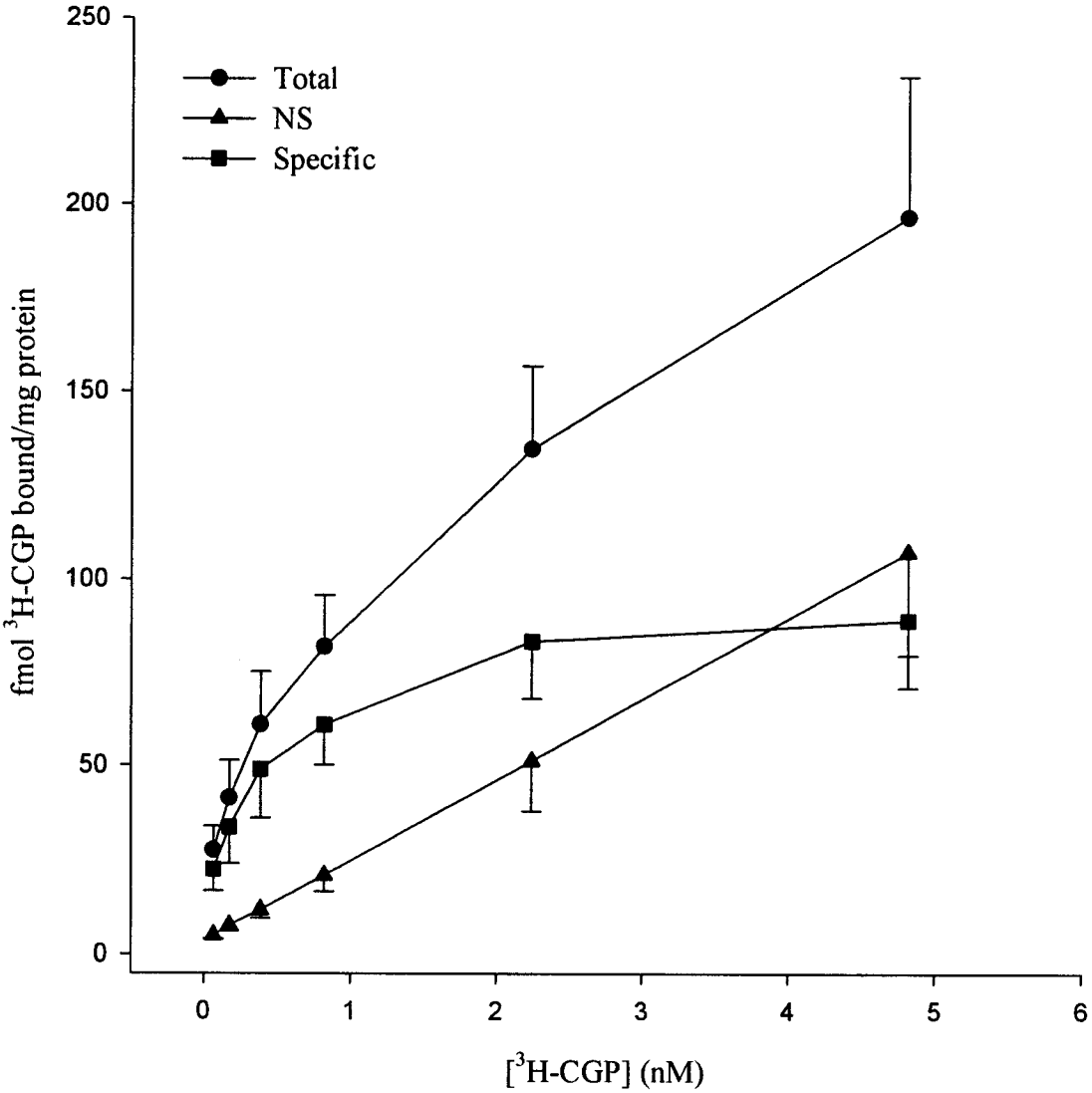


Figure 3.1. Association curve of ^{3}H-CGP-12177 binding to semi-purified gill membranes from rainbow trout. Membranes (75-100 µg protein/50 µl) were incubated for 60 min with increasing concentrations of ^{3}H-CGP-12177 in either the absence (Total binding, circle) or presence of 10 µM alprenolol (ALP, non-specific (NS) binding, triangle). Specific binding (square) was determined by subtracting NS from Total binding. Values are means + or – SEM (n = 5).

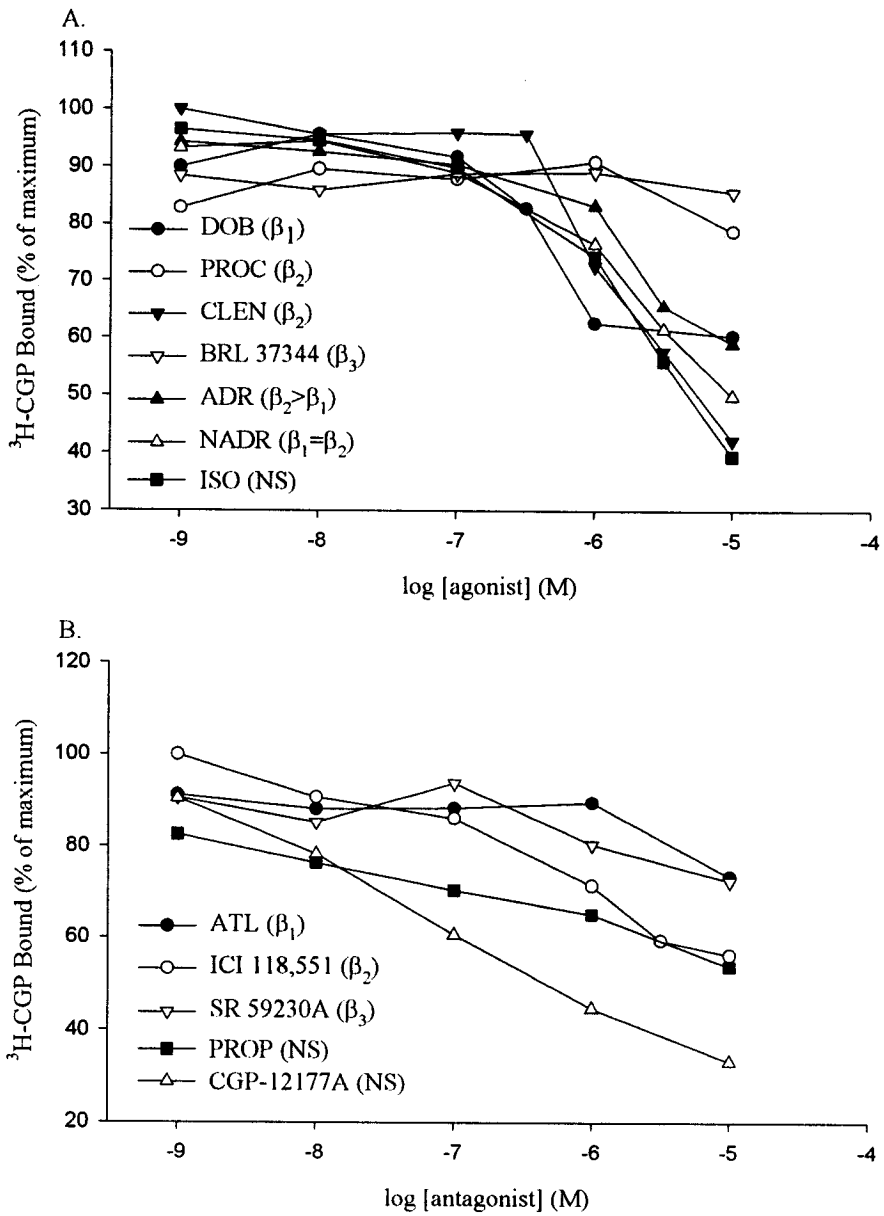


Figure 3.2. Competitive displacement of ^{3}H-CGP-12177 by selective agonists (A) and antagonists (B). Semi-purified membranes were incubated for 60 min with a saturating concentration (2 nM) of ^{3}H-CGP with increasing concentrations of the unlabelled ligands. Data represent means (n = 4–6). SEM for each individual data point was approximately ± 5% and was omitted for clarity.

Table 3.1. Inhibitory binding constants (K_i) for adrenergic agonists and antagonists in rainbow trout gill membranes. Binding assays used a saturating concentration (2 nM) of ^{3}H-CGP-12177. Values are means ± SEM (n = 4-6). Ligand selectivity is presented in parentheses. N/A indicates 50% inhibition was not achieved (up to 10 μM).

AGONIST	K_i (M)
Dobutamine (β_1)	$4.42 \pm 1.74 \times 10^{-7}$
Procaterol (β_2)	$2.21 \pm 0.29 \times 10^{-6}$
Clenbuterol (β_2)	$6.19 \pm 4.89 \times 10^{-7}$
BRL 37344 (β_3)	N/A
Adrenaline ($\beta_2 > \beta_1$)	$5.21 \pm 0.99 \times 10^{-7}$
Noradrenaline ($\beta_1 = \beta_2$)	$6.07 \pm 2.11 \times 10^{-7}$
Isoproterenol (non-selective β)	$1.87 \pm 0.59 \times 10^{-7}$
ANTAGONIST	
Atenolol (β_1)	N/A
ICI 118,551 (β_2)	$1.60 \pm 1.11 \times 10^{-6}$
SR 59230A (β_3)	$7.85 \pm 1.81 \times 10^{-7}$
CGP-12177A (non-selective β)	$7.36 \pm 3.70 \times 10^{-9}$
Propranolol (non-selective β)	$1.94 \pm 0.97 \times 10^{-8}$

fish exposed to normocarbic water (Fig. 3.3). However, there was a slight trend towards a decrease in B_{max}. There was no significant difference for either binding parameter between the preliminary binding study results and either control (normocarbia) or hypercarbia exposed trout.

3.4 Discussion

This study pharmacologically characterized the β-AR in rainbow trout gill. To my knowledge, only one other study has examined β-AR binding in gill membranes from a teleost. Steevens *et al.* (1996) using the hydrophobic antagonist ^{3}H-dihydroalprenolol (^{3}H-DHA) found a K_d of 0.93 nM and B_{max} of 94 fmol/mg protein in a crude membrane preparation from brown bullhead (*Ameiurus nebulosus*). Unfortunately, the authors did not specify whether binding best fit a one or a two site model nor was any attempt made to pharmacologically subtype this β-AR. The lower values of K_d and B_{max} reported here for rainbow trout compared with those of the brown bullhead is most likely the combination of different ligands being used and different species. Binding of ^{3}H-CGP to crude plasma membrane in rainbow trout gill was saturable (Fig. 3.1) and showed high affinity. Scatchard analysis was linear (data not shown) suggesting one class of binding sites was present in this membrane preparation. Molecular evidence, however, supports the presence of both a β_{3a}-AR and to a lesser extent, a β_2-AR in rainbow trout gill tissue (Nickerson *et al.*, 2001; 2003). Thus, one cannot rule out the possibility of two binding sites possessing similar affinities for ^{3}H-CGP that cannot be distinguished kinetically. A single class of ^{3}H-CGP binding sites was

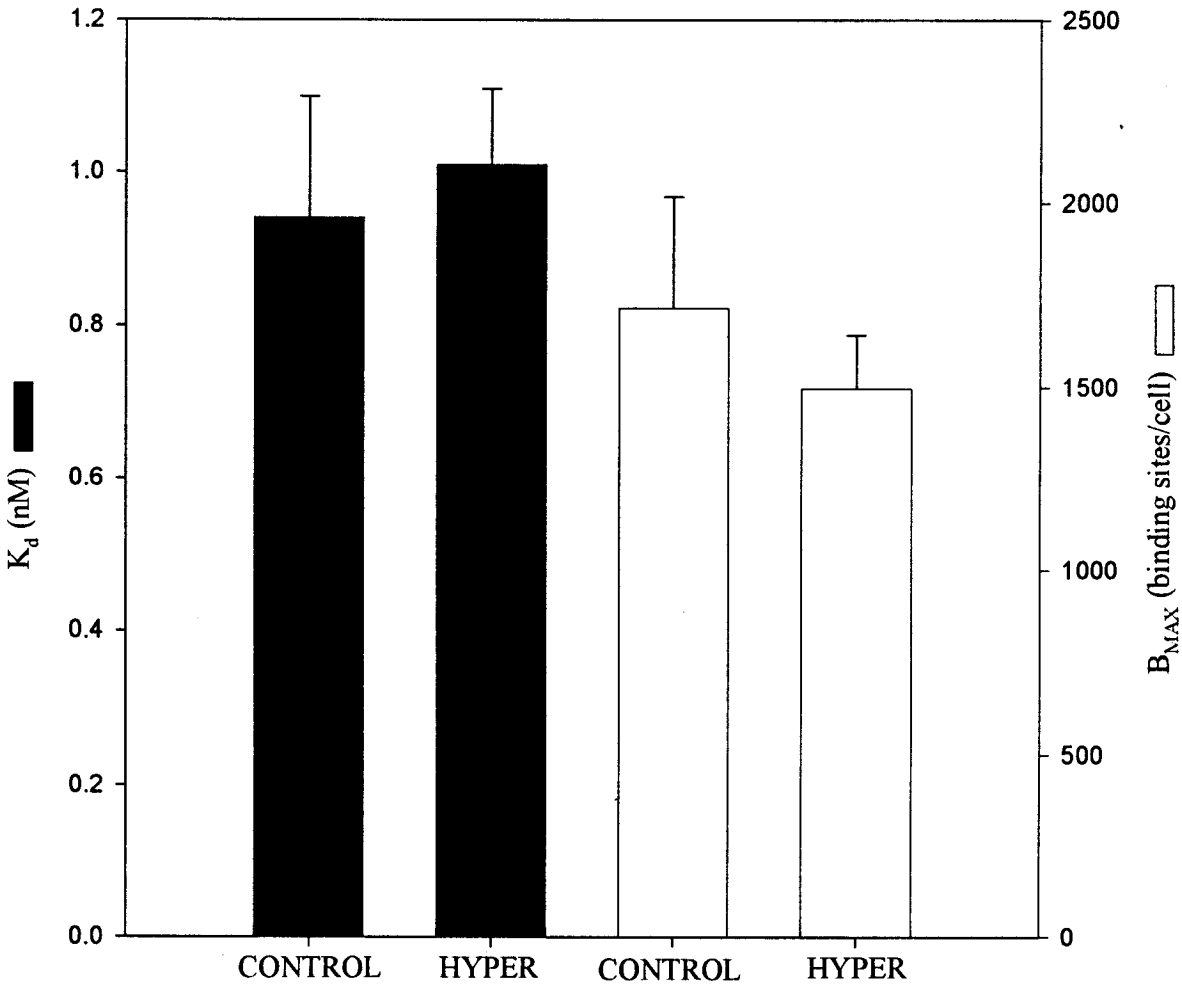


Figure 3.3. Binding parameters of isolated gill cells from rainbow trout exposed to normocarbic (CONTROL) and hypercarbic (HYPER – 8 mmHg P_{CO_2}) water for 2 d. There were no significant differences for either binding affinity (K_d, black bars) or number of binding sites (B_{MAX}, white bars). Data represent means + SEM (n = 6).

also seen by Lortie and Moon (2003) in red muscle membrane preparations from rainbow trout where molecular evidence also supports the existence of both β_2- and β_{3a}-ARs (Nickerson *et al.*, 2001; 2003).

Competitive displacement studies (Fig. 3.2; Table 3.1) support the presence of a gill β-AR with a novel pharmacology that does not fall into one of the three classical mammalian β-AR categories. Displacement of ^{3}H-CGP with the various agonists supports a pharmacology that most closely resembles the mammalian β_1-AR. The observed potency order of ISO>ADR=NADR supports a β_1-AR classification (Lomasney *et al.*, 1995). This is further supported by DOB being the most effective of the synthetic selective agonists. However, this pharmacology is not supported by the antagonist competition studies. The β_3-AR selective SR 59230A was more effective than the β_2-antagonist ICI 118,551 while the β_1-selective antagonist ATL was ineffective at displacing ^{3}H-CGP. While this may suggest the presence of a β_3-like AR, the non-selective PROP was much more effective than SR 59230A. As the mammalian β_3-AR is known to have a low affinity for this antagonist (Summers and McMartin, 1993), caution must be exercised in attempting to pharmacologically classify this β-AR into one of the three classical mammalian subtypes. Pharmacological characterization of the rainbow trout β_{3a}-AR is difficult due to the co-existence of the β_2-AR in all of the same tissues (Nickerson *et al.*, 2001; 2003). Depending on the relative expression levels of the two receptors, pharmacological characterization using mammalian agonists and antagonists will most probably result in an atypical classification as seen in this study as well as that by Lortie and Moon (2003) in red muscle. But, similarity to another β-AR subtype is also possible. As seen in gill tissue, molecular studies in trout heart also show a high expression of the β_{3a}-AR with the β_2-AR showing a much lower level of expression (Nickerson *et al.*, 2001; 2003).

Gamperl *et al.* (1994b) suggested a pharmacological classification of a β_2-AR in rainbow trout heart based on the potency order of ISO>ADR>NADR as well as displacement of ^{3}H-CGP by the selective β_2-antagonist ICI 118,551 with no inhibition by the β_1-antagonist ATL. Similarly in this study, ATL was ineffective and ICI 118,551 displaced ^{3}H-CGP binding with comparable effectiveness with a K_i of 160 nM versus 300 nM from Gamperl *et al.* (1994b). Comparable K_i's were also seen for ISO and ADR with the relative effectiveness of NADR being the only discrepancy between the two displacement studies. Thus, accurate pharmacological characterization of the trout β_{3a}-AR would require either the development of a highly selective antagonist for the trout β_2-AR to block those binding sites or by transfection studies.

Exposing rainbow trout to hypercarbia for 2 d resulted in no significant difference for either the K_d or B_{max} values of the β-AR of subsequently isolated gill cells (Fig. 3.3). However, it is important to note that this gill cell isolation included both PVC and MR gill cells. It is not currently known if β-ARs are found on only one or both cell types or if there are any β-AR subtype differences between the two cell types. As previously stated, there is molecular evidence for both the β_{3a}-AR and the β_2-AR in gill tissue (Nickerson *et al.*, 2001; 2003). Therefore, the possibility that a decrease in one subtype was offset by an increase in another cannot be excluded. It is also possible that the absence of a significant change in either kinetic parameter may have resulted because the plasma CAs levels were not sufficiently elevated to a magnitude to cause a change in the number of binding sites. Although significantly elevated relative to control fish, Perry and Reid (2002) reported mean plasma ADR and NADR levels of only 17.4 and 5.4 nM, respectively after 20 min at this level of hypercarbia. Similarly, Reid and Perry (1995) found no change in red blood cell β-

AR numbers after 48 h of hypoxia with significantly elevated mean plasma ADR and NADR levels less than 20 and 10 nM, respectively.

While gill tissue possesses mRNA for both βARK and β-arrestin, the β_{3a}-AR possesses only two potential phosphorylation sites in its short cytoplasmic tail (Nickerson *et al.*, 2004). Thus, as is commonly seen with mammalian β_3-ARs (Summers and McMartin, 1993; Marullo *et al.*, 1995), it is possible the β_{3a}-AR is relatively resistant to down-regulation. Gamperl *et al.* (1994b; 1998) were unable to find any changes in rainbow trout heart β-ARs after either repeated CA injections or after exposure to moderate hypoxia, respectively. β-AR regulation does not likely occur at the mRNA level as Nickerson *et al.* (2004) did not find any changes in either rainbow trout gill β_{3a}- or β_2-AR mRNA after either hypoxia or cortisol treatment.

In contrast to this study, a decrease in the number of gill β-ARs due to stressor exposure was previously reported by Steevens *et al.* (1996). These authors found 60% fewer gill β-ARs with no significant difference in K_d in brown bullhead chronically exposed to polycyclic aromatic hydrocarbons (PAH) compared to bullheads from a reference site. Unfortunately, plasma CAs were not measured and therefore it cannot be determined if the reduction seen resulted from chronically elevated plasma CA levels or to something else. No significant changes in β-AR numbers were found by Steevens *et al.* (1996) in either heart or brain membrane preparations but, it is not known whether those two tissues possess the same subtype of β-AR that is found in the gill of the brown bullhead.

Pharmacological characterization of the rainbow trout gill β-AR supports an atypical classification of an AR that appears resistant to changes in binding parameters with stressor exposure. It is important to note that this was only a preliminary investigation in examining

the gill β-AR. Future studies will need to determine the precise location and function of the β_{3a}- and β_2-ARs as well as the role of βARK and β-arrestin in gill tissue.

Chapter 4: Black bullhead hepatic β-adrenoceptor regulation

4.1 Introduction

Exposures to stressors ranging from biological (eg. predation) to environmental (eg. hypoxia) are common to most teleost fish species resulting in the release of the catecholamines (CA), adrenaline (ADR) and noradrenaline (NADR) from chromaffin cells in the posterior cardinal vein of the head kidney. These two hormones function by binding to members of the large superfamily of G-protein coupled receptors (GPCR) known as adrenoceptors (AR) in various target tissues. Activation of the ARs leads to a signal transduction cascade resulting in cardiorespiratory and metabolic modifications to ameliorate the effects of the exposure to the stressor (reviewed by Fabbri *et al.*, 1998a; Reid *et al.*, 1998).

However, an attenuation of the response in order to prevent overstimulation is often seen within minutes of exposure of the ARs to ADR or NADR by a process known as desensitization. With continued agonist exposure, receptors are sequestered inside the cell where they will be either recycled back to the cell membrane or be degraded. This reduction in the total number of cellular receptors is referred to as down-regulation. These regulatory processes have been well studied in mammalian systems and especially for the β_2-AR, which shows a high degree of desensitization relative to the other β-AR subtypes (Lohse, 1993). Regulation of the β-ARs is accomplished by phosphorylation of the receptor in the third intracellular loop and cytoplasmic tail by a member of the G-protein receptor kinase (GRK) family known as β-adrenoceptor kinase (βARK). This permits binding of another protein

termed β-arrestin resulting in an uncoupling of the receptor from the G-protein (Hein and Kobilka, 1995; Marullo *et al.*, 1995; Nickerson *et al.*, 2004).

Relatively little is known about the regulation of the ARs in non-mammalian vertebrates. It has not yet been demonstrated if the same regulatory mechanisms seen in mammalian systems are also involved in β-AR regulation of lower vertebrates such as teleost fish but, desensitization and decreases in the number of β-AR binding sites were previously reported in rainbow trout (*Onchorhynchus mykiss*) erythrocytes (Thomas *et al.*, 1991; Gilmour *et al.*, 1994; Perry *et al.*, 1996) and hepatic tissue (Chapter 2). As well, a β-arrestin was cloned in trout red blood cells (Jahns *et al.*, 1996). In addition, mRNA for both β-arrestin and βARK was detected in other tissues from rainbow trout including gill, kidney, liver, white muscle and spleen (Nickerson *et al.*, 2004). Black bullhead hepatic tissue is known to contain both α_1- (Fabbri *et al.*, 1995b) and β-ARs (Fabbri *et al.*, 1992) where they mediate glucose production by increasing glycogenolysis (Brighenti *et al.*, 1987b). The present study examines the *in vitro* and *in vivo* effect of agonist and stressor exposure on hepatic β-AR binding characteristics and function in the black bullhead (*Ameiurus melas*). additionally, the receptor was sequenced to determine if the phosphorylation sites in the third intracellular loop and cytoplasmic tail that are known to be involved in the regulation of mammalian β-ARs are also present in the hepatic β-AR of the black bullhead.

4.2 Materials and Methods

4.2.1 Animals

Black bullheads, of either sex, weighing approximately 200-250 g were obtained from a cultured stock at the State University of New York (SUNY), Brockport, NY and

transported to the University of Ottawa. Fish were maintained in a fiberglass holding tank (1275 l) of well-aerated, dechloraminated city of Ottawa tap water at 18 ± 1°C with a constant 12L:12D photoperiod. Fish were fed to satiation 3 times a week (Purina cage catfish chow, Purina Mills Inc., St Louis, MO). Experiments were conducted from November to January (ISO exposure), February to March (competition binding), April to May (1st air exposure study) and November to January (2nd air exposure study) and commenced between 10 am and 2 pm. Animals were cared for and treated in accordance with the guidelines from the Canadian Council on Animal Care.

4.2.2 Agonist exposure

The effect of agonist exposure on AR properties was examined using hepatocytes isolated for primary cell culture. Fish were randomly sampled from the holding tank and killed by a quick blow to the head. Hepatocytes were isolated using the method of Mommsen *et al.* (1994) as described in Chapter 2 for rainbow trout, except cells were resuspended in Hanks' medium containing essential (2 mg/100 ml) and non-essential amino acids (1 mg/100 ml) (Gibco). Isolated hepatocytes (4 million cells/ml) were plated in 60 x 15 mm polystyrene plates (Falcon Primaria) and incubated for approximately 40 h at 10°C. At this point, plates either received isoproterenol (ISO) (final concentration 10 μM) containing 100 μM ascorbic acid to prevent agonist oxidation or saline containing only ascorbic acid. Twenty-four hours later, the medium was removed and cells were resuspended in Hanks' without ascorbic acid or ISO. Cells were centrifuged twice for 2 min at 120 g and resuspended in Hanks' medium with essential and non-essential amino acids at a concentration of 40-100 mg/ml and used for binding assays (see below).

4.2.3 Air exposure

Bullheads were exposed to air for 1 h (in the lab sink) as an acute stress. Two different studies of this stressor were completed, however both studies used identical air-exposure protocols. The first was conducted in the spring and examined the effect of air-exposure on ADR and NADR-stimulated glucose production. The second study was conducted in the autumn and examined the effect of the air-exposure on receptor binding parameters. In both studies just prior to the air-exposure, a small blood sample (approximately 500 µl) was taken by caudal puncture with a heparinized syringe to determine pre-stress plasma parameters. After 1 h, fish were killed by a quick blow to the head and another caudal puncture blood sample was taken. Blood was centrifuged for 1 min at 12,000 g, plasma removed and frozen in liquid nitrogen before storing at -70°C for future analysis. Non-air-exposed controls were collected directly from the main holding tank and sampled as described for post-air-exposed fish. A mid-ventral incision was made to expose the liver, the hepatic portal vein was canulated for hepatocytes isolation as described above except hepatocytes were resuspended in 2% BSA for binding assays and glucose production. Cell viability was verified by trypan blue exclusion using a hemacytometer (American Optical) for all hepatocyte isolations and was greater than 80 % for all experiments.

4.2.4 Binding assays

Binding parameters for the β-ARs in all studies were determined with the hydrophilic β-antagonist ((-)-4-(3-t-butylamino-2-hydroxypropoxy)-[5,7-^{3}H]benzimidazol-2-one) ^{3}H-CGP-12177 (^{3}H-CGP; Amersham, specific activity 45.0 Ci/mmol). Fifty µl of hepatocytes

(containing 2 – 5 mg of cells depending upon the study) were incubated for 30 min at room temperature (19 ± 1°C) in a final volume of 150 µl in the presence of varying concentrations of ^{3}H-CGP as previously reported (Chapter 2). Non-specific binding was determined in the presence of 10 µM alprenolol (ALP). Specific binding was the difference between total and non-specific binding. For displacement studies to characterize the hepatic β-AR, 50 µl of freshly isolated hepatocytes from fish randomly sampled from the main holding tank were incubated with a saturating concentration of ^{3}H-CGP (2 nM) alone or with increasing concentrations (1 nM to 10 µM) of selective and non-selective agonists and antagonists for 30 min at room temperature. All adrenergic agonists and antagonists used were purchased from Sigma Chemical Co. (St. Louis, MO). Incubations were terminated with 4 washes of ice-cold 0.9% NaCl using a cell harvester (Brandel 24R). Membranes were collected on borosilicate filters (#32 Mandel Scientific) and placed in scintillation vials containing 4 ml scintillation cocktail (Safety-Solve; Research Products International, Mount Prospect, IL). Vials were left in the dark at room temperature for at least 24 h before radioactivity was determined using either a Packard 2500 TR or a Beckman Coulter LS 6500 liquid scintillation counter. Binding parameters (K_d, K_i, and B_{max}) were determined using the EBDA and LIGAND computer programs (Munson and Rodbard, 1980).

4.2.5 Glucose production

One hundred microlitres containing approximately 8-10 mg of cells from either control or air-exposed fish were placed in 1.5 ml polypropylene microcentrifuge tubes and allowed to rest for 2 h to allow activated glycogen phosphorylase (GPase) activities to return to basal levels (Moon *et al.*, 1999). In the first air-exposure study, cells either received saline

or increasing concentrations of ADR or NADR (final concentrations 0.5 nM to 5.0 μM) for 1 h. Assays were terminated by addition of perchloric acid (PCA, final concentration approximately 1.1 %). Cells were then frozen at -20°C for future analysis. Upon thawing, cells were centrifuged for 2 min at 12,000 g (Eppendorf 5415C) and the supernatants were assayed for glucose enzymatically using hexokinase and glucose-6-phosphate dehydrogenase (Moon *et al.*, 1999).

4.2.6 Plasma parameters

In the air exposure experiments, plasma samples from both control (not air-exposed) and air-exposed were analyzed for catecholamines, cortisol, glucose, and lactate in the first study and catecholamines, cortisol, glucose, and blood O_2 in the second study. Plasma catecholamines, ADR and NADR were measured as described in Perry *et al.* (1996). Plasma cortisol was measured with a commercial radioimmunoassay (RIA) kit (ICN Pharmaceuticals, Orangeburg, NY) as described in Dugan and Moon (1998). Both plasma glucose (Moon *et al.*, 1999) and plasma lactate (Bergmeyer, 1983) were measured enzymatically. Blood O_2 was measured using an OXYCON™ blood oxygen content analyzer (Cameron Instrument Co., Port Aransas, TX).

4.2.7 Molecular characterization

RNA was originally isolated from a pool of black bullhead tissues, including the liver, for PCR amplification using the degenerate primers BetaUni 5' GGIAAYBTIYTIGTXAT and BetaUni 3' CCARCAIARISWRAAIRYICCCATDAT (Nickerson *et al.*, 2001). The primers Bh B5'1 CAAGACCCCTACGCCATAAAGT and Bh

B3'1 TAATTTGGATGGCCTTCGTC were then designed from the sequence of the clones obtained for initial PCR amplification of the hepatic β-AR. To sequence the hepatic β-AR, total cellular RNA was isolated from frozen liver samples (QIAGEN) according to the manufacturer's protocol. The concentration and quality of RNA was verified by spectrophotometry and gel electrophoresis. Prior to cDNA synthesis, RNA was treated with DNase I. Random hexamer primed cDNA was synthesized using the 1st Strand cDNA Synthesis Kit for RT-PCR (Roche Molecular Biologicals, Laval, QC). A preliminary round of PCR amplification using the primers Bh B5'1 and Bh B3'1 was done to produce a clone from which gene specific primers for 3' RACE could be designed. cDNA synthesis for 3' RACE was primed with the 3' amplification primer (3AP). The first round of PCR amplification used the bullhead gene specific primer BH3RACE31 TGATCCGAAATGCTGTGATT and the abridged universal amplification primer (AUAP). A second round of semi-nested PCR was then done using the bullhead gene specific primer BH3RACE32 CACCAAAGTGTTTGCCATTG and AUAP. The primers ADRFW2 CTSGCNTGYGCNGAYCTSGTNATG and ADRREV1 AANGGSAGCCARCASAGNGTRAANGT were used in attempt to amplify a β_2-AR. PCR amplifications used the following cycles of denaturing, annealing and extension: 1 X 2 min at 94 °C, 35 X (30 or 45 sec at 94 °C, 30 or 45 sec at 53 – 55 °C, 60, 90 or 120 sec at 72 °C), 1 X 10 min at 72 °C. Potential phosphorylation residues of serine, threonine, and tyrosine in the third intracellular loop and cytoplasmic tail was accomplished using NETPHOS 2.0 (http://www.cbs.dtu.dk/services/NetPhos/) (Blom *et al.*, 1999).

4.2.8 Phylogenetic analysis

The partial amino acid sequence of the bullhead hepatic β-AR was aligned with GenBank sequences of the three β-AR subtypes using the selected organisms from Nickerson *et al.* (2003). Maximum likelihood phylogenetic analysis was performed using PUZZLE version 4.0.2 (Thompson *et al.*, 1994) as described in Nickerson *et al.* (2001; 2003).

4.2.9 Statistical analysis

Statistical differences were evaluated using appropriate tests depending upon the experiment (Sigma Stat version 2.0, Jandel Scientific). These included unpaired t-tests and 2-way repeated measures ANOVA, followed by an appropriate multiple comparison test. Level of significance was taken to be $P<0.05$.

4.3 Results

4.3.1 Pharmacological characterization

Displacement studies using selective and non-selective mammalian agonists and antagonists suggest the black bullhead hepatic β-AR pharmacologically most closely resembles the mammalian β_2-AR (Fig. 4.1). Agonists and antagonists that are selective or have higher affinity for the β_2-AR were more effective at inhibiting ^{3}H-CGP binding than the β_1-AR selective ligands. In addition, the potency order of ISO>ADR>NADR observed also supports a β_2-AR classification (Lefkowitz, 1978). Inhibitory constants (K_i) for the ligands are shown in Table 4.1.

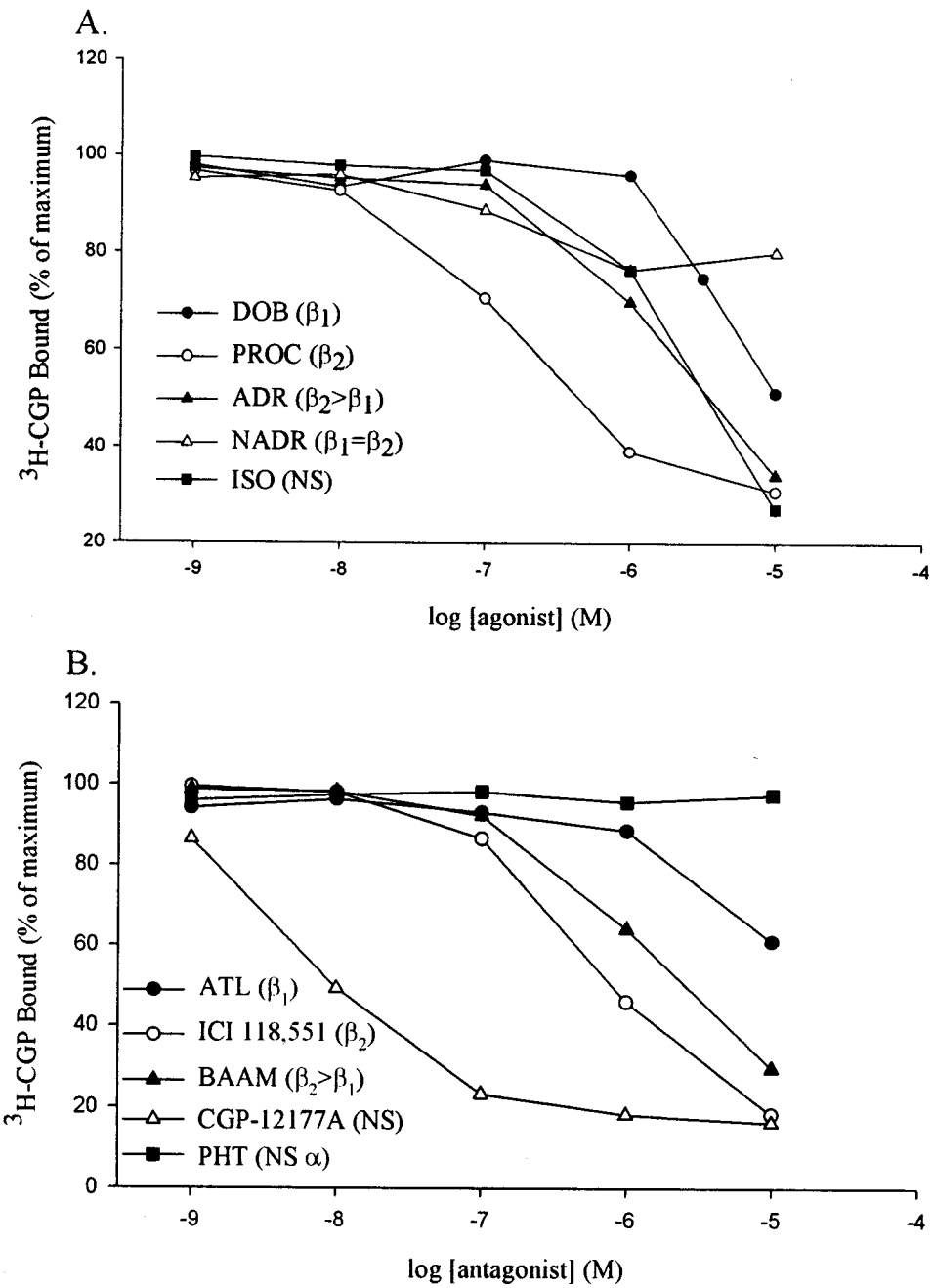


Figure 4.1. Competitive displacement of ^{3}H-CGP-12177 by selective and non-selective agonists (A.) and antagonists (B.). Isolated hepatocytes were incubated for 30 min with a saturating concentration of ^{3}H-CGP (2 nM) with increasing concentrations of the non-labeled ligands. Values represent means (n = 3-6). Variation (SEM) for individual data points was less than 5% and is omitted for clarity.

Table 4.1. Adrenergic agonist and antagonist inhibition constants (K_i) for isolated black bullhead hepatocytes ARs. Binding assays used a saturating concentration (2 nM) of ^{3}H-CGP-12177. Values are means ± SEM (n = 3-6) calculated from individual curves as indicated in Fig. 4.1. N/A indicates 50% inhibition was not achieved (up to 10 μM).

Agonist	Ki (M)
Dobutamine (β_1)	N/A
Procaterol (β_2)	$1.12 \pm 0.11 \times 10^{-7}$
Adrenaline ($\beta_2 > \beta_1$)	$1.51 \pm 0.62 \times 10^{-6}$
Noradrenaline ($\beta_2 = \beta_1$)	N/A
Isoproterenol (non-selective β)	$6.08 \pm 1.51 \times 10^{-7}$
Antagonist	
Atenolol (β_1)	N/A
ICI 118,551 (β_2)	$7.01 \pm 2.19 \times 10^{-7}$
BAAM[a] ($\beta_2 > \beta_1$)	$1.20 \pm 0.24 \times 10^{-6}$
CGP-12177A (non-selective β)	$5.64 \pm 0.95 \times 10^{-9}$
Phentolamine (non-selective α)	N/A

[a] **Bromoacetyl alprenolol menthane**

4.3.2 Agonist-exposure

Hepatocytes were isolated and subjected to a 40 h culture period, followed by a 24 h exposure to the non-selective β-agonist ISO (10 μM). There were no significant differences seen for either affinity (K_d) or number of cell surface binding sites (B_{max}) (Fig 4.2). However, trends existed for an increase in K_d and a decrease in B_{max}. These trends are more evident with Scatchard analysis which also supports a single class of saturable ^{3}H-CGP binding sites is present on the cell surface of black bullhead hepatocytes (Fig. 4.3).

4.3.3 Air-exposure

Glucose production was significantly elevated above their respective 1 h saline controls at 5 nM ADR and 50 nM NADR in hepatocytes isolated from control and 1 h air-exposed bullheads. In both groups, NADR maximally elevated glucose production approximately 3-fold above their respective controls. However, ADR maximally elevated glucose production 3.1-fold in the control group and 2.6-fold in the air-exposed group. Significant differences in glucose production between the two groups were observed in the 1 h controls, from 5 nM to 5 μM ADR and from 50 nM to 5 μM NADR (Fig. 4.4). In the second study, no significant differences were observed with either kinetic parameter after the 1 h air-exposure (Fig. 4.5), although the same trends were observed as with the 24 h exposure to ISO (Fig. 4.2).

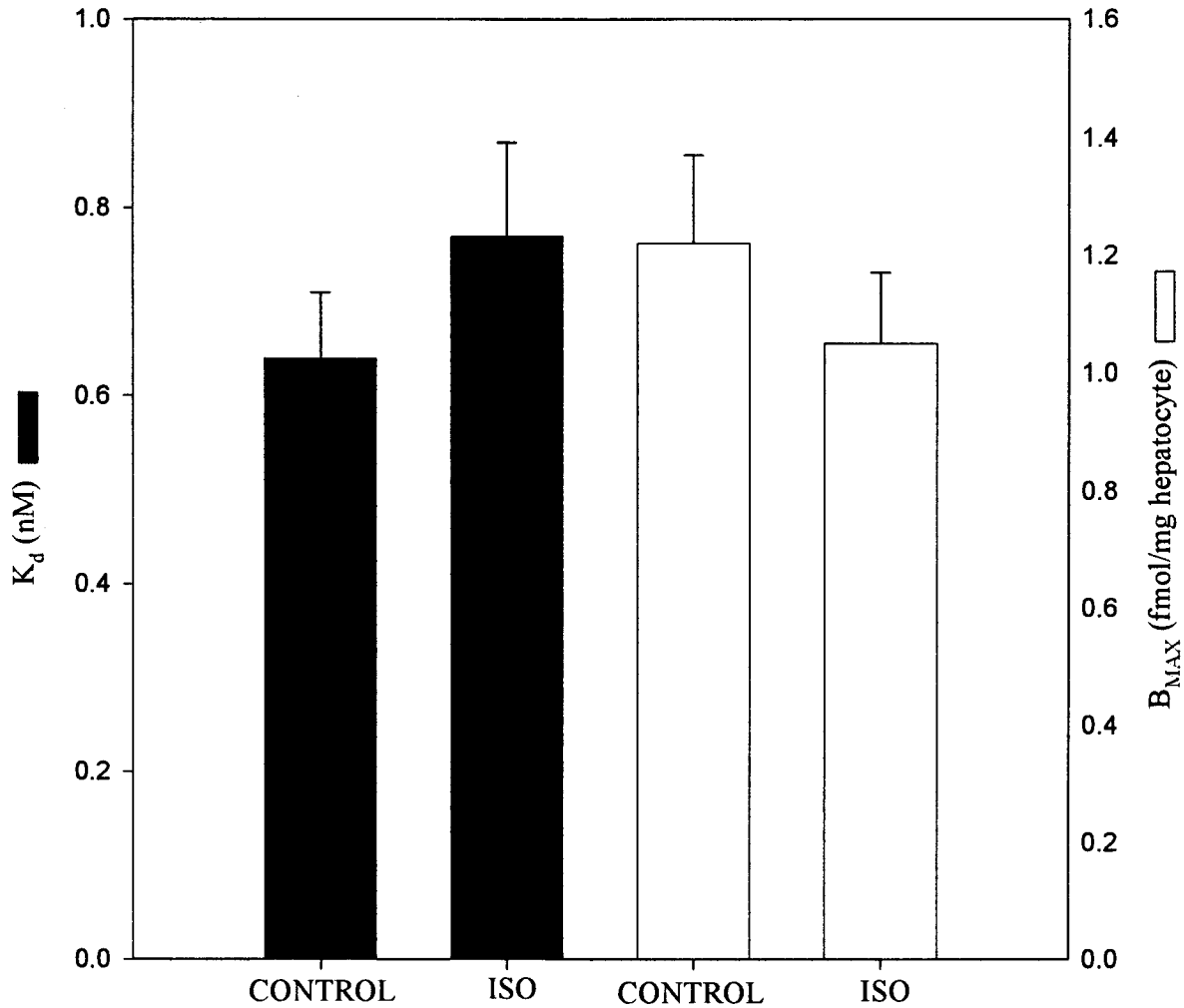


Figure 4.2. Binding parameters of hepatocytes incubated for 24 h with 10 μM of the β-agonist isoproterenol (ISO) or saline (CONTROL). No significant differences were observed for either binding affinity (K_d, black bars) or number of binding sites (B_{MAX}, white bars). Data represent means + SEM (n = 5).

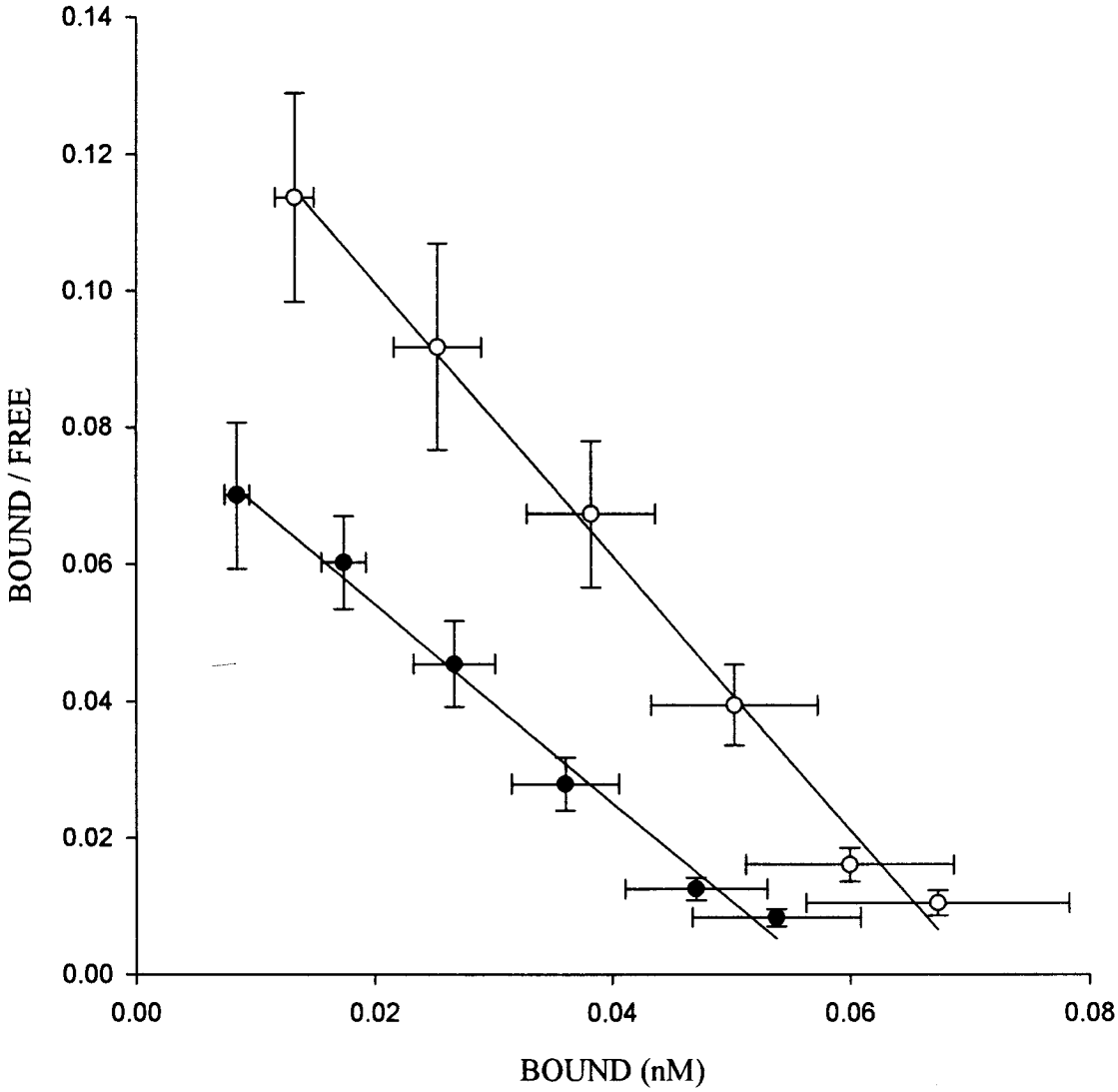


Figure 4.3. Scatchard plot of hepatocytes incubated for 24 h with 10 μM of the β-agonist isoproterenol (ISO, black circles) or saline (CONTROL, white circles). Data were calculated using EBDA (Munson and Rodbard, 1980). Points are means ± SEM (n = 5).

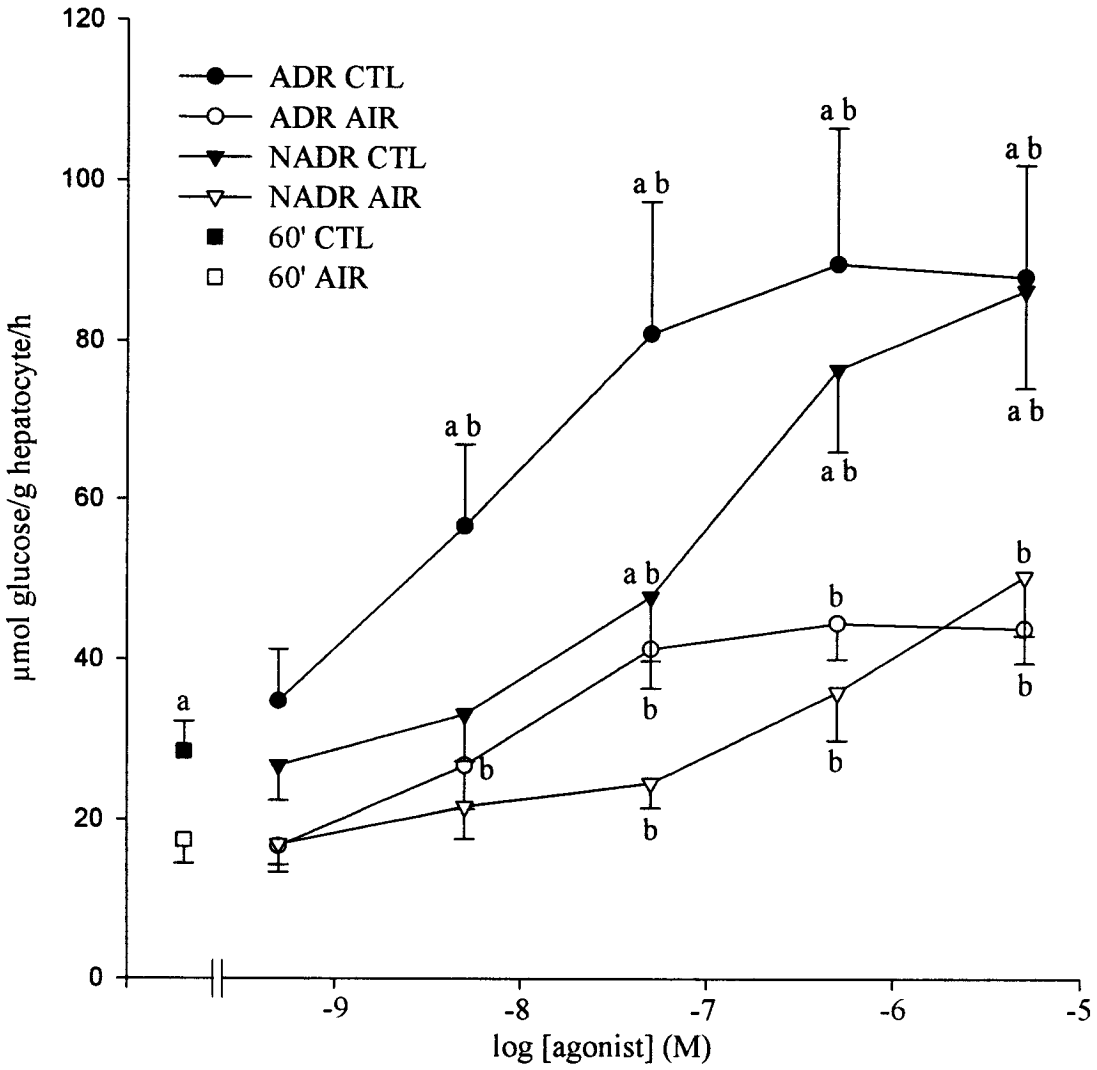


Figure 4.4. Glucose production in isolated black bullhead hepatocytes from fish exposed to air for 1 h (AIR, white symbols) and control fish not exposed to air (CTL, black symbols). Approximately 8-10 mg of cells were incubated for 1 h with increasing concentrations of either adrenaline (ADR) or noradrenaline (NADR) or Hanks' saline (60' CTL). Values are means + or – SEM (n = 5-7). Significant difference from air-exposure is represented by the letter a, and from the respective saline control by the letter b (2-way repeated measures ANOVA followed by Tukey, $P<0.05$).

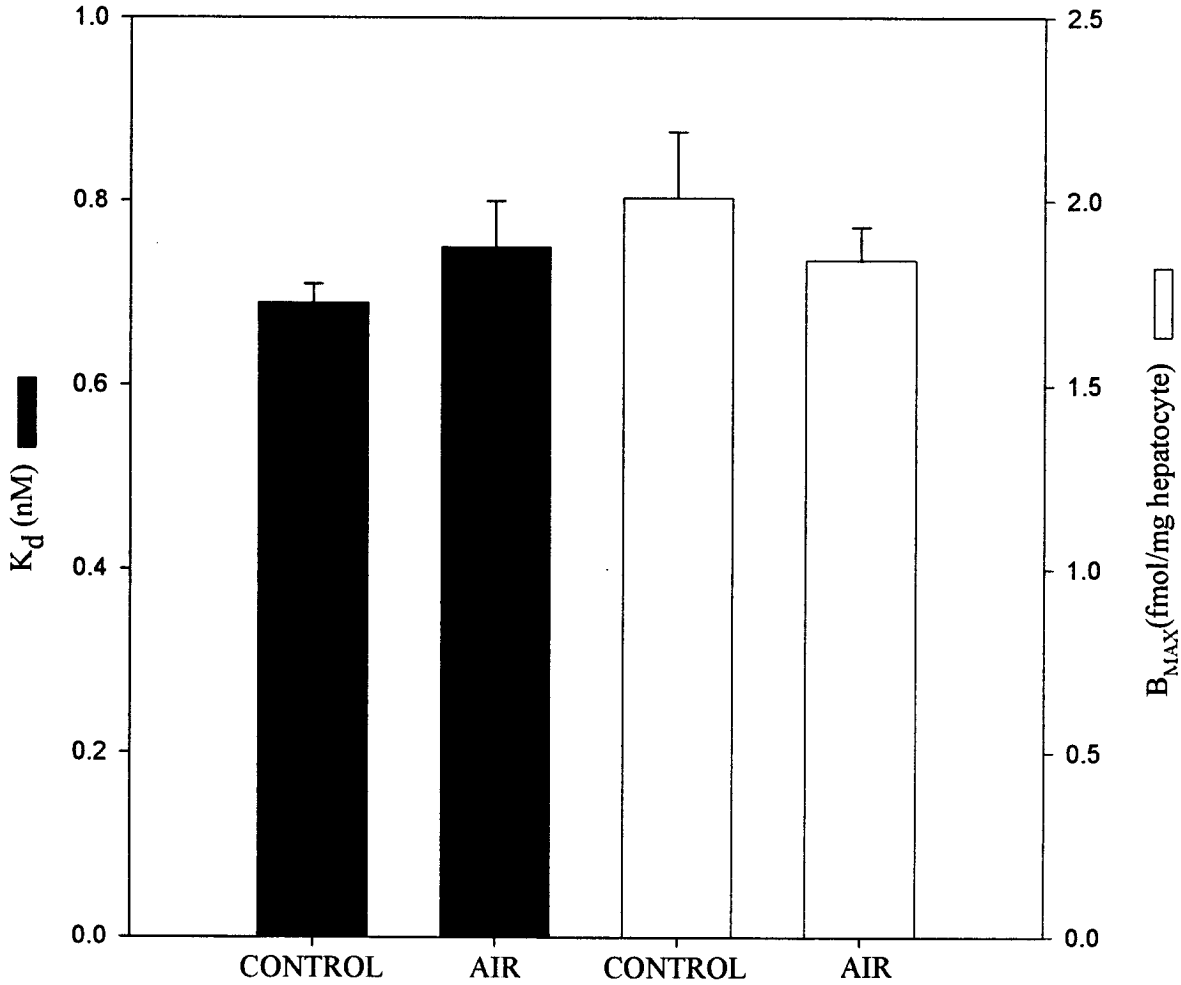


Figure 4.5. Binding parameters of hepatocytes from black bullheads exposed to air for 1 h (AIR) or in non-air-exposed controls (CONTROL). No significant differences were observed for either binding affinity (K_d, black bars) or number of binding sites (B_{MAX}, white bars). Values are means + SEM (n = 4-5).

The exposure of bullheads to air for 1 h resulted in significantly elevated levels of plasma ADR and NADR as well as plasma cortisol, glucose and lactate in the first study (Table 4.2). In the second study (Table 4.3), despite the strong trend in ADR and cortisol, only NADR and glucose were significantly elevated above the pre air-exposure levels. No significant differences were found for any blood parameter between controls and pre air-exposed fish in either study. NADR was the predominant CA released into the circulation following air-exposure in both studies. Blood O_2 was not significantly decreased after the 1 h air-exposure although a strong trend was seen (Table 4.3).

4.3.4 Molecular characterization

Using RT-PCR, a partial β-AR sequence of 379 amino acids was obtained that spanned the region from the second transmembrane domain to the carboxy terminal tail (Fig.4.6). Phylogenetic analysis of this partial sequence places this hepatic β-AR with the teleost β_3-ARs at the base of the mammalian β_3-AR group (Fig. 4.7). The partial sequence obtained of the black bullhead hepatic β-AR showed the highest level of amino acid similarity with the rainbow trout β_3-ARs with 65% and 60% identity with the β_{3a}-AR and β_{3b}-AR, respectively (see Nickerson *et al.*, 2003).

Prediction of putative phosphorylation sites of this partial β-AR sequence using NetPhos 2.0 (Blom *et al.*, 1999) identified three potential sites within the second G-protein binding domain of the third intracellular loop with another four in close proximity. The cytoplasmic carboxyl tail was found to possess an additional seven potential sites (Fig. 4.6).

Table 4.2. Plasma catecholamines, glucose, lactate and cortisol in control and air-exposed black bullheads (Spring). Values represent means ± SEM (n = 5-7). Significant difference from pre air-exposure and control indicated by the letter [a] (one-way ANOVA, $P<0.05$).

Plasma	Control	Pre air-exposure	Post air-exposure
Adrenaline (nM)	0.68 ± 0.51	1.06 ± 0.33	11.9 ± 3.78[a]
Noradrenaline (nM)	2.28 ± 0.58	1.82 ± 0.74	52.8 ± 17.6[a]
Glucose (mM)	3.09 ± 0.39	3.76 ± 0.73	7.22 ± 0.69[a]
Lactate (mM)	2.19 ± 0.39	2.08 ± 0.36	6.17 ± 0.45[a]
Cortisol (ng/ml)	1.68 ± 0.18	<1.5[b]	18.7 ± 4.50[a]

[b] All samples were below the 1.5 ng/ml detection limit of RIA kit

Table 4.3. Plasma catecholamines, glucose, cortisol and total blood oxygen in control and air-exposed black bullheads (late Fall/early Winter). Values represent means ± SEM (n = 4-5). Significant difference from pre air-exposure and control indicated by the letter [a] (one-way ANOVA, $P<0.05$).

Plasma	Control	Pre air-exposure	Post air-exposure
Adrenaline (nM)	2.70 ± 1.41	2.12 ± 0.51	9.58 ± 3.46
Noradrenaline (nM)	10.0 ± 4.82	3.77 ± 1.53	47.3 ± 10.5[a]
Glucose (mM)	3.64 ± 0.49	3.49 ± 0.52	14.0 ± 1.76[a]
Cortisol (ng/ml)	4.84 ± 3.03	2.06 ± 0.37	16.30 ± 5.24
Blood O_2 (mM)	0.97 ± 0.20	1.52 ± 0.47	0.47 ± 0.20

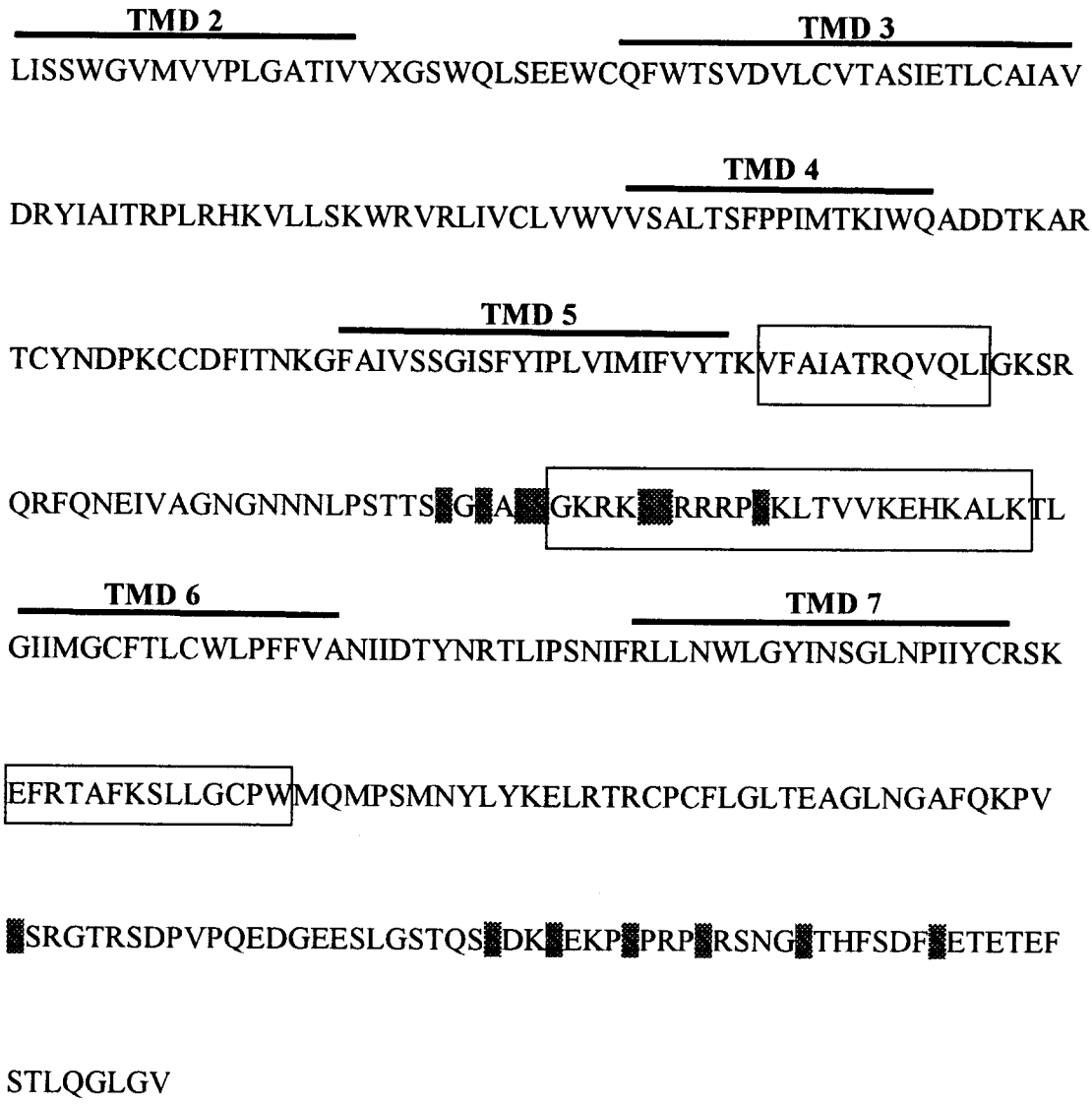
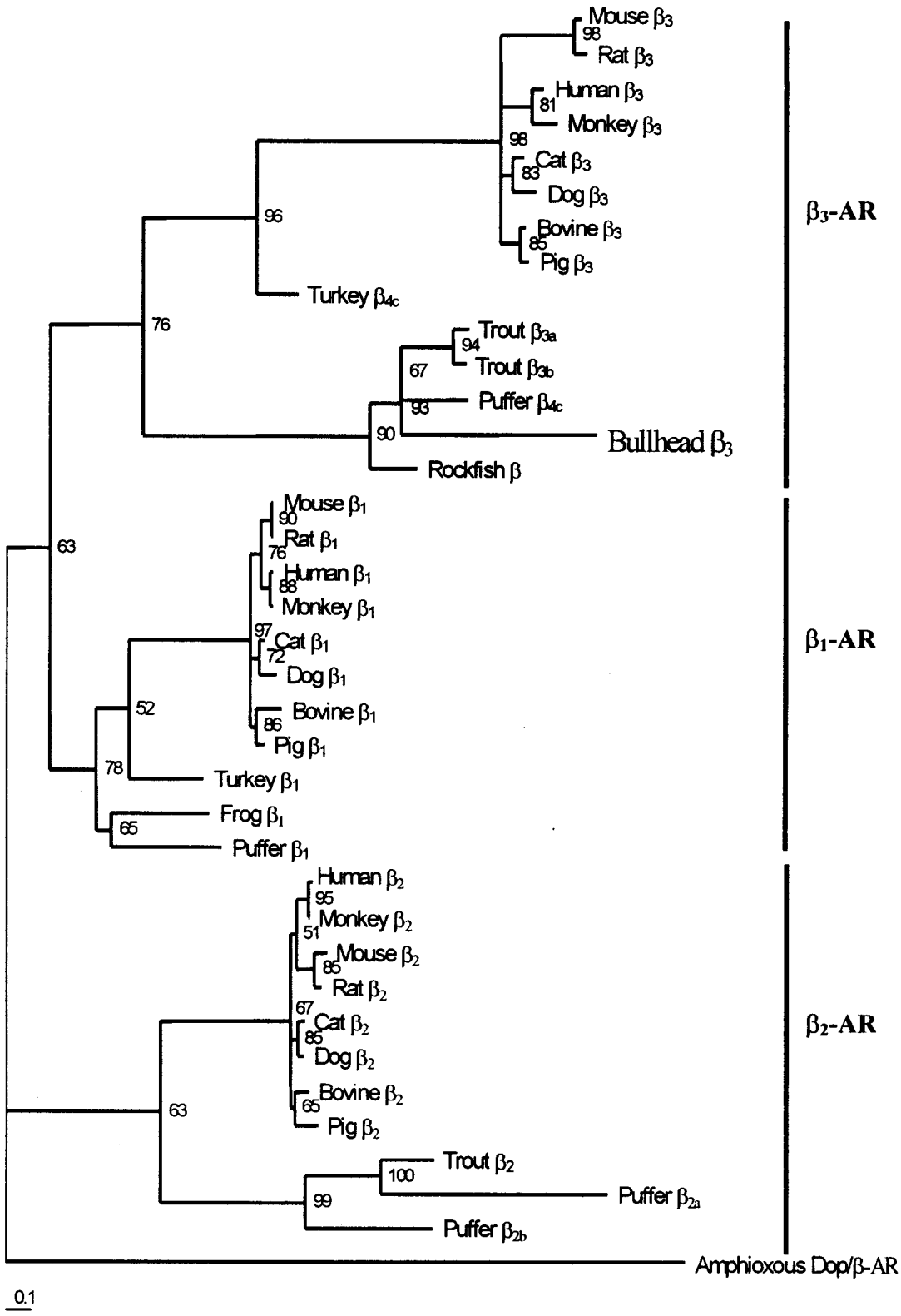


Figure 4.6. Partial amino acid sequence of the black bullhead hepatic β-AR. The transmembrane domains (TMD) are indicated by a heavy line and the G-protein binding domains by boxes. The highlighted serine residues are potential phosphorylation sites identified by NetPhos 2.0 (Blom *et al.*, 1999).

Figure 4.7. Maximum likelihood tree of the three major vertebrate β-AR subtypes, including the black bullhead hepatic β-AR, inferred from amino acid alignment analysis. Branch lengths are scaled to represent relative number of amino acid substitutions. Percent support values (out of 1000 puzzling steps) are presented at the nodes. The amphioxus dopamine/β-AR sequence was used as an outgroup


Mouse β3
98
Rat β3
Human β3
81
Monkey β3
98
Cat β3
83
Dog β3
96
Bovine β3
85
Pig β3
Turkey β4c
76
Trout β3a
94
Trout β3b
67
Puffer β4c
93
90
Bullhead β3
Rockfish β
β3-AR
Mouse β1
90
Rat β1
76
Human β1
88
Monkey β1
63
97
Cat β1
72
Dog β1
52
Bovine β1
86
Pig β1
78
Turkey β1
Frog β1
65
Puffer β1
β1-AR
Human β2
95
Monkey β2
51
Mouse β2
86
Rat β2
67
Cat β2
86
Dog β2
Bovine β2
65
63
Pig β2
Trout β2
100
Puffer β2a
99
Puffer β2b
β2-AR
Amphioxous Dop/β-AR
0.1

4.4 Discussion

These studies examined how an agonist *in vitro* and an acute stressor *in vivo* affected hepatic β-AR binding parameters and function in the black bullhead. After 24 h of exposure to 10 μM ISO, there was a trend but no significant decrease in the number of cell surface receptors (Fig. 4.2). This is in contrast to Lima (1996) who reported more than an 80% decrease in the number of cell surface β_2-ARs in L6 myoblasts after a 24 h exposure to 10 μM ISO. In addition, a comparable decrease was observed by Barak and Caron (1995) after approximately 16 h of 20 μM ISO exposure for β_2-ARs transfected into Chinese hamster ovary (CHO) cells. Scatchard analysis of ^{3}H-CGP binding was linear suggesting a single class of receptors present on the cell surface of black bullhead isolated hepatocytes (Fig. 4.3). This is in agreement with one class of binding sites also found in the livers of rainbow trout (Fabbri *et al.*, 1995a; Dugan and Moon, 1998), European eel, *Anguilla anguilla* (Fabbri *et al.*, 2001), and channel catfish, *Ictalurus punctatus* (Finkenbine *et al.*, 2002). In contrast, Scatchard analysis by Fabbri *et al.* (1992) supported the existence of a low and a high affinity binding site in purified hepatic membranes of black bullhead using the lipophilic antagonist [^{3}H]dihydroalprenolol (^{3}H-DHA). These authors (Fabbri *et al.*, 1992) suggested the possibility that a single receptor site was present that possessed different affinities for ^{3}H-DHA, which is one possible explanation for the observed discrepancy between my study and that of Fabbri *et al.* (1992). However, as was seen with the rainbow trout gill binding study (Chapter 3), the possibility of two binding sites possessing similar affinities for ^{3}H-CGP that cannot be separated kinetically should not be excluded.

Competition studies using mammalian selective agonists and antagonists supports the idea that the black bullhead hepatic β-AR most closely resembles pharmacologically the

mammalian β_2-AR subtype (Fig. 4.1; Table 4.1). This is in agreement with the presence of a β_2-AR in the liver of other vertebrate species (see Chapter 1 for references). While Fabbri *et al.* (1992) did not specifically characterize the black bullhead liver β-AR using selective ligands, these authors did find ADR more effective than NADR at inhibiting ^{3}H-DHA binding, indicative of a pharmacological β_2-AR subtype being present in the hepatic membranes of this species. However, the partial molecular sequence obtained of the black bullhead hepatic β-AR does not corroborate this pharmacological classification as this receptor was found to phylogenetically group with the β_3-ARs (Fig. 4.6). This discrepancy is most likely due to using mammalian subtype selective agonists and antagonists in a non-mammalian system. Previous competition studies with ^{3}H-CGP in rainbow trout ventricular punches have also supported a pharmacological β_2-AR classification (Gamperl *et al.*, 1994b). As was also seen in my study (Fig. 4.1), Gamperl *et al.* (1994b) found the selective β_2-AR antagonist ICI 118,551 was more effective than the β_1-AR antagonist ATL and a potency order of ISO>ADR>NADR. However, molecular evidence in the rainbow trout heart supports a higher expression of the β_{3a}-AR (Nickerson *et al.*, 2003) with the β_2-AR being expressed to a much lesser extent (Nickerson *et al.*, 2001). Therefore, unless ligands selective for specific β-AR subtypes in teleosts and other non-mammalian vertebrates are developed, further discrepancies between pharmacological and molecular classifications are probable.

In mammals, the β_3-ARs are primarily expressed in adipose tissue where they mediate a thermogenic response by increasing lipolysis (Strosberg, 1997). The two β_3-ARs reported in the rainbow trout by Nickerson *et al.* (2003) were more widely distributed, although the β_{3b}-AR was localized exclusively to the red blood cell. Tissue-specific roles for

the β_{3a}-AR have yet to be elucidated. Neither of the two β_3-AR subtypes were detected in the liver of the rainbow trout using the highly selective RNase protection assay (Nickerson *et al.*, 2003). To my knowledge, this is the first report of a β_3-AR in hepatic tissue from any vertebrate species. As mentioned above, the β_2-AR is the subtype that is reported in the liver of other vertebrate species. Given the pharmacology observed, the presence of a β_2-AR cannot be excluded. However, all attempts to find a β_2-AR gene in black bullhead hepatic tissue have been unsuccessful to date.

Although the pharmacology of the bullhead hepatic β-AR is ISO>ADR>NADR, exposing black bullheads to air for 1 h resulted in a preferential release of NADR over ADR; NADR was significantly elevated from both pre-air exposure levels in both experiments (Tables 4.2 and 4.3). The levels of plasma ADR and NADR attained were comparable to those reported in carp after a 15 min air stress (Fuchs and Albers, 1988). While ADR is the predominant plasma catecholamine in most teleosts (Perry and Randall, 1992; Gamperl *et al.*, 1994a), preferential release of NADR has also been reported in other teleosts including the channel catfish (Finkenbine *et al.*, 2002) and carp, *Cyprinus carpio* (Fuchs and Albers, 1988; Van Raaij *et al.*, 1996). The lack of a decrease in the number of binding sites after a 1 h air-exposure may be related to the low plasma ADR levels achieved. The mean plasma ADR levels of 11.9 and 9.58 nM (Tables 4.2 and 4.3) obtained during the two studies, respectively, could be considered as an unstressed level in other species of teleosts where values in excess of 300 nM are reported (Gamperl *et al.*, 1994a; Wendelaar Bonga, 1997). As the black bullhead hepatic β-AR has a higher affinity for ADR over NADR (Fig. 4.1), plasma ADR levels may not have reached a sufficient level to result in a significant decrease in the number of cell surface receptors (Fig. 4.5). Gamperl *et al.* (1998) found no change in myocardial β-

ARs in rainbow trout exposed to hypoxia for 6 h. While significantly elevated above control levels, plasma ADR and NADR did not increase above 10 and 30 nM, respectively. Similarly, Reid and Perry (1995) found no change in rainbow trout red blood cell β-ARs after a 48 h hypoxia exposure and concluded plasma CA levels were insufficiently elevated as to cause a reduction in receptor number. Thus, the level of plasma CAs achieved may be of more importance than duration of the stressor at inducing desensitization and down-regulation of β-ARs.

Evidence for a pharmacological β_2-AR subtype being present is also supported by glucose production studies. At physiological concentrations, ADR was more effective than NADR at stimulating glucose production in hepatocytes from both control and air-exposed hepatocytes (Fig. 4.4). Similar to this study, Brighenti *et al.* (1987b) found concentrations of 10 μM ADR and NADR were equally effective at stimulating glucose production in black bullhead hepatocytes. However, even at pharmacological doses, Ottolenghi *et al.* (1988) and Brighenti *et al.* (1987a) found ADR more effective than NADR at activating adenylyl cyclase (ACase) and stimulating cAMP production, respectively. Therefore, caution must be taken when attempting to pharmacologically subtype a receptor based on the relative potencies of ADR and NADR.

Air-exposure resulted in a moderate 16% decrease in glucose production (Fig. 4.4). As no antagonists were used, it cannot be determined if this decrease is strictly due to a desensitization of the β-ARs. Fabbri *et al.* (1999) demonstrated α_1-AR mediated glucose release in perifused hepatocytes from the black bullhead. In contrast to this study, Manzl *et al.* (2002) found no change in either α_1- or β-AR mediated glucose production in goldfish (*Carassius auratus*) isolated hepatocytes exposed to chemical anoxia for 1 h. Further study

is required to determine if α_1-ARs maintain CA-stimulated glucose production during β-AR desensitization. The decrease in glucose production due to the air-exposure is contrary to Wright *et al.* (1989) who examined the effect of hypoxia on hepatic glycogenolysis in rainbow trout. Plasma glucose was significantly elevated after air-exposure (Tables 4.2 and 4.3) but no significant change was observed in hypoxia-exposed rainbow trout. Despite significantly elevated plasma CA levels, there was no apparent desensitization of the hepatic β-AR as hypoxia significantly elevated ADR-stimulated GPase a activities. Desensitization of the β-ARs in mammals occurs as a result of phosphorylation of the receptor by βARK and the subsequent binding of β-arrestin leading to an uncoupling from the G-protein. While βARK and β-arrestin mRNA have been detected in rainbow trout hepatic tissue (Nickerson *et al.*, 2004), the trout β_2-AR possesses few potential phosphorylation sites relative to its mammalian counterpart (Nickerson *et al.*, 2001). Therefore, it is not unexpected that there might not be any observed desensitization of the hepatic β-AR response after hypoxia exposure. No desensitization was seen in hepatic ACase activity after chronic exposure to adrenergic agonists (Chapter 2). In contrast, the black bullhead hepatic β-AR was found to possess several potential phosphorylation sites in both the G-protein binding domain of the third intracellular loop as well as in the tail (Fig. A1). However, it is not currently known if βARK and β-arrestin are present in the liver of this species. It should be noted that the absence of βARK and β-arrestin does not exclude phosphorylation by either PKA or PKC. The possible role these potential sites may have in the regulation of the black bullhead hepatic β-AR requires further attention. Despite the presence of several potential phosphorylation sites in the third intracellular loop and cytoplasmic tail, the black bullhead hepatic β-AR appears to be resistant to changes in binding kinetics. The contradictory

physiological and molecular data suggest the presence of a novel hepatic β-AR subtype in this species that is regulated differently from the other β-AR subtypes.

Chapter 5: General Summary

Regulation of cell signaling is of vital importance in order to prevent overstimulation. The mechanisms involved have been well studied in mammalian systems. In particular, the processes of desensitization and down-regulation are well characterized for the large superfamily of receptors that couple to G-proteins (GPCR), which includes the adrenoceptors (AR). Mammals express three different β-ARs, each with a different capacity to desensitize and down-regulate. From this group, the β_2-AR is the best understood and is known to exhibit a high degree of desensitization and down-regulation and is often used as a model for GPCR regulation. Desensitization and down-regulation of β-ARs in mammalian systems involves phosphorylation of specific amino acids in key regions of the third intracellular loop and cytoplasmic tail. This is accomplished by a member of the GRK family termed βARK, which permits binding of another protein known as β-arrestin resulting in an uncoupling of the AR from the G-protein. Phosphorylation of ARs may also occur by the second messenger dependent protein kinases, PKA and PKC (Lohse, 1993; Marullo *et al.*, 1995; Ferguson, 2001).

Few studies, however, have examined β-AR regulation in non-mammalian systems. The studies presented in this thesis examined the regulation of β-ARs in the liver and gills of rainbow trout and in the liver of the black bullhead. As in mammals, rainbow trout express three different β-ARs, one β_2-AR and two that phylogenetically group with the mammalian β_3-ARs, termed the β_{3a}- and β_{3b}-ARs (Nickerson *et al.*, 2001; 2003). Previous studies of β-AR regulation in rainbow trout focused on the β_{3b}-AR, which is localized only in the red blood cell (RBC) (Nickerson *et al.*, 2003). In fact, a teleost β-arrestin, which shows high

sequence similarity to its mammalian counterpart, was first cloned in the RBCs of the rainbow trout (Jahns *et al.*, 1996) and subsequently has been found to be expressed in several other tissues in the rainbow trout (Nickerson *et al.*, 2004).

Desensitization in RBCs was first shown by Thomas *et al.* (1991) after a 48 h hypoxia exposure. Thomas *et al.* (1991) found from 60% to total inhibition in the adrenergic response of the β-AR coupled Na^+/H^+ exchanger (βNHE). However, the authors did not examine receptor binding characteristics and as such, this diminished responsiveness may not be due solely to a decrease in receptor number. Jahns *et al.* (1996) were not able to show any change in the cellular distribution of β-arrestin after a 30 min exposure of the RBCs to 0.55 μM isoproterenol (ISO) despite desensitization of the βNHE. As a result, Jahns *et al.* (1996) suggested the possibility of β-arrestin involved in the regulation of the βNHE itself. In addition, neither Perry and Reid (1992) nor Reid and Perry (1995) were able to find any changes in either affinity (K_d) or number of AR binding sites (B_{max}) in trout RBCs with hypoxia exposure. However, Thomas *et al.* (1991) reported plasma catecholamine (CA) levels of more than an order of magnitude higher than the latter two studies. The requirement for plasma CAs to be sufficiently elevated as to cause a change in binding site numbers was supported by Gilmour *et al.* (1994). Using mini-osmotic pumps, Gilmour *et al.* (1994) achieved plasma ADR levels in excess of 100 nM and found a 33% decrease in the number of RBC surface binding sites after 3 d of elevated CA exposure. Perry *et al.* (1996) found a 20% decrease 24 h after daily chasing to exhaustion for 7 d. Plasma CA levels immediately after chasing were in excess of 100 nM and therefore it is probable that Perry *et al.* (1996) would have observed a larger per cent decrease in RBC ARs had binding been

examined sooner as plasma CA levels were no longer significantly elevated and receptor numbers were most likely starting to return to control levels.

Of the three β-AR genes in rainbow trout, the β_2-ARs are the most ubiquitously distributed, as they were expressed in 7 out of the 8 tissues examined (Nickerson *et al.*, 2001). However, few studies have examined β_2-ARs in teleosts in tissues other than the liver and I am aware of only two studies to date that have attempted to examine β_2-AR regulation. Gamperl *et al.* (1994b) were unable to detect any changes in the number of ^{3}H-CGP binding sites in rainbow trout ventricular punches 1 h after five bolus CA injections over a 6 h period. Similarly, Gamperl *et al.* (1998) found no changes after a 6 h moderate hypoxia exposure. However, while Gamperl *et al.* (1994b) found the rainbow trout heart β-AR to be exclusively of a pharmacological β_2-AR subtype, molecular evidence supports the predominant presence of the β_{3a}-AR subtype (Nickerson *et al.*, 2003). It is possible the β_2-AR and β_{3a}-AR cannot be separated kinetically in rainbow trout heart tissue with ^{3}H-CGP and therefore, if the total surface receptor number remained unchanged, significant changes in the specific subtypes would not be detectable without subtype selective ligands. Therefore, unless subtype-specific ligands, can be developed for the different trout β-ARs, pharmacological experiments alone examining *in vivo* regulation in tissues where more than one subtype is present may not be able to detect any changes in receptor binding parameters. However, transfection studies could be used to examine the *in vitro* regulation of a particular β-AR subtype.

While the β_2-AR pharmacology reported by Gamperl *et al.* (1994b) in the heart is not supported by the molecular evidence of Nickerson *et al.* (2003), there is little doubt as to the β-AR subtype present in the liver of rainbow trout. Previous binding studies support a single

class of ^{3}H-CGP binding sites (Fabbri *et al.*, 1995; Dugan and Moon, 1998) pharmacologically classified as a β_2-AR (Reid *et al.*, 1992). This classification was later supported by both molecular and pharmacological studies (Nickerson *et al.*, 2001). Nickerson *et al.* (2003) were unable to detect the presence of either β_{3a}- or β_{3b}-AR mRNA in rainbow trout hepatic tissue using the highly sensitive RNase protection assay, again supporting a single receptor type. In contrast to the pharmacological β_2-AR of Gamperl *et al.* (1994b; 1998), stressor exposure can alter the binding characteristics of the hepatic β_2-AR (Chapter 2). Both chasing to exhaustion and prolonged exposure to the β_2-agonist clenbuterol (CLEN) significantly reduced the number of ^{3}H-CGP binding sites by 27% and 33%, respectively (Figs. 2.2B and 2.3B) without any change in affinity (Figs. 2.2A and 2.3A). This was the first study to examine the regulation of and the first to report a decrease in the number of binding sites for a confirmed β_2-AR in a teleost. However, the exact mechanisms behind this loss of binding sites remain uncertain. The loss does not appear to be due to a change in receptor mRNA levels as no significant difference was found between SHAM and CLEN-fed fish (Fig. 2.4). Similarly, Nickerson *et al.* (2004) found no differences in rainbow trout β-AR mRNA expression levels in gill, heart, and liver after hypoxia or cortisol treatments. Nickerson *et al.* (2004) did find βARK and β-arrestin mRNA in rainbow trout hepatic tissue, however, potential phosphorylation sites within the 3rd intracellular loop are lacking and only 1 of 3 potential sites in the cytoplasmic tail aligned with similar sites in mammalian β_2-ARs (Nickerson *et al.*, 2001). The presence of potential phosphorylation sites in the tail does support the possibility of βARK/β-arrestin mediated regulation of the hepatic β_2-AR. Future studies would need to examine receptor

phosphorylation to verify the role of βARK and β-arrestin in mediating changes in the number of binding sites.

The observed lack of any stressor-induced changes in trout cardiac β-AR numbers by Gamperl *et al.* (1994b; 1998) was supported by no change in gill β-AR binding kinetics after a 2 d hypercarbia exposure (Fig. 3.3). Both tissues contain predominantly the β_{3a}-AR subtype, which possesses a short cytoplasmic tail with only two potential phosphorylation sites (Nickerson *et al.*, 2003) and therefore may also be relatively resistant to down-regulation. The three mammalian β-ARs display different potential phosphorylation site profiles and display different sensitivities to desensitization and down-regulation (Marullo *et al.*, 1995). Similarly, the three rainbow trout β-ARs also display different phosphorylation site profiles (Nickerson *et al.*, 2001; 2003) and appear to display different sensitivities to regulation. As previously stated, the rainbow trout β_2-AR possesses three potential carboxy terminal sites and changes in plasma membrane numbers have been shown (Figs. 2.2B and 2.3B). The β_{3b}-AR possesses eight potential sites in the cytoplasmic tail and six in the third intracellular loop and in addition, the RBC expresses higher levels of βARK and β-arrestin relative to other tissues (Nickerson *et al.*, 2004). Although the reported percent decreases in the number of binding sites is comparable between the β_2- and β_{3b}-AR, desensitization of the RBC β-AR has been previously demonstrated (Thomas *et al.*, 1991). No change in ACase activity after prolonged CLEN exposure was seen in hepatic membranes (Fig. 2.5). Similarly, there was no effect of a 1 day chase to exhaustion on cAMP production in isolated hepatocytes (data not shown). This discrepancy in the sensitivity to desensitization between the β_2- and β_{3b}-AR may be a result of the β_2-AR not possessing any potential phosphorylation sites in the third intracellular loop.

The study of the gill β-AR (Chapter 3) was the first attempt to pharmacologically characterize and examine the regulation of the β_{3a}-AR. Pharmacology of the gill β-AR (Fig. 3.2; Table 3.1) was comparable to that of the heart β-AR (Gamperl *et al.*, 1994b) with the relative affinity for NADR being the only discrepancy and to the red muscle β-AR (Lortie and Moon, 2003), two other tissues that also express the β_{3a}-AR (Nickerson *et al.*, 2003). While Gamperl *et al.* (1994b) found a pharmacological resemblance to the mammalian β_2-AR, displacement studies in gill and red muscle support an atypical pharmacology. However, more subtype selective ligands were used in the gill study and that of Lortie and Moon (2003) compared to Gamperl *et al.* (1994b). The difficulty in attempting to pharmacologically classify fish ARs using mammalian ligands was also demonstrated by Jozefowski and Plytycz (1998) who examined β-AR binding in goldfish kidney and spleen cells and found each tissue possessed a single class of ^{3}H-CGP binding sites. While Nickerson *et al.* (2001; 2003) showed molecular evidence for the presence of only a β_2-AR in these tissues from rainbow trout, Jozefowski and Plytycz (1998) using subtype selective ligands were unable to pharmacologically classify the β-AR from either tissue into one of the three classic mammalian subtypes.

In contrast to the rainbow trout, the subtype identity of the black bullhead hepatic β-AR is questionable (Chapter 4). Despite the fact that displacement studies support a β_2-AR pharmacological classification (Fig. 4.1; Table 4.1) as is seen in rainbow trout hepatic tissue (Fig. 2.1; Table 2.1), the partial molecular sequence obtained phylogenetically groups the bullhead hepatic AR with the β_3-ARs (Fig. 4.6). This β_3-AR classification is in contrast to the β_2-AR classification from other vertebrate species (see Chapter 1 for references) and to my knowledge the first report of a β_3-AR in hepatic tissue. β_{3a}- and β_{3b}-AR mRNA were not

detectable in rainbow trout liver (Nickerson *et al.*, 2003). Using quantitative ribonuclease protection assays, McNeel and Mersmann (1999) could not detect a β_3-AR transcript in porcine hepatic tissue. However, McNeel and Mersmann (1999) did find a 45:55 proportion of β_1- to β_2-ARs and therefore, the β_2-AR is not the sole β-AR subtype that may be found in vertebrate hepatic tissue. In fact, a partial β-AR sequence obtained from hepatic tissue of the copper rockfish (*Sebastes caurinus*) also phylogenetically groups with the β_3-ARs (see Fig. 4.6; Nickerson, Mommsen and Moon, unpublished results). While the bullhead hepatic β-AR is preferentially stimulated by ADR (Fig. 4.4), the rockfish hepatic β-AR possesses a higher affinity for NADR (Danulat and Mommsen, 1990) supporting this molecular classification. Although attempts to amplify a β_2-AR in hepatic tissue using degenerate β_2-AR primers were unsuccessful, the presence of a β_2-AR in the liver of the black bullhead cannot be excluded. It is not known if this β_3-AR mRNA translates into a functional G-protein coupled β_3-AR. β_2-AR mRNA in the bullhead liver may have a very low level of expression that could not be amplified by RT-PCR. Nickerson *et al.* (2001) used RNase protection assays rather than less sensitive Northern blots to examine β_2-AR tissue expression in rainbow trout.

Regardless of the pharmacology, in contrast to the rainbow trout hepatic β_2-AR, stressor exposure did not modify binding characteristics of the bullhead hepatic β-AR (Figs. 4.2 and 4.5). This may be the result of more than one subtype being present that could not be distinguished with ^{3}H-CGP. Fabbri *et al.* (1992) found two classes of ^{3}H-DHA binding sites in purified black bullhead hepatic membranes. The lack of a change in the number of binding sites after air-exposure (Fig. 4.5) is likely related to an insufficient elevation of plasma CAs (Table 4.3). As noted above, studies examining trout red blood cell β-AR

binding parameters found no significant changes when plasma CAs were not sufficiently elevated. Of particular interest, is the preferential release of NADR over ADR, which is contrary to most species of teleosts that have been studied (Randall and Perry, 1992; Gamperl *et al.*, 1994a). The bullhead hepatic β-AR displays low affinity for NADR (Fig. 4.1; Table 4.1) and thus, it is not unexpected that significantly elevated NADR would not significantly impact β-AR numbers. Further, no change in B_{max} was seen after a 24 h incubation of bullhead hepatocytes with 10 μM ISO (Fig. 4.2). This result would appear contradictory as NetPhos 2.0 (Blom *et al.*, 1999) found seven potential phosphorylation sites in both the third intracellular loop as well as the tail (Fig. A1). Moreover, a moderate desensitization in CA-mediated glucose production was shown after exposing bullheads to air for 1 h to simulate exposure to an acute stressor (Fig. 4.4). It is not currently known if black bullhead hepatic tissue possesses βARK and β-arrestin or if any of the potential phosphorylation sites were involved in mediating the observed desensitization. However, attempts to examine desensitization *in vitro* by exposing isolated hepatocytes to ADR or NADR found no difference in CA-mediated glucose production between hepatocytes pre-exposed to either ADR, NADR or saline controls (data not shown). Similarly, Perry and Reid (1992) as well as Reid and Perry (1995) were unable to find desensitization in red blood cells exposed *in vitro* to hypoxia.

The work presented in this thesis was the first to comparatively examine differences in regulation for different β-AR subtypes in two species of teleost fishes. Significant findings include the first demonstration of stressor induced reduction in β_2-ARs plasma membrane binding sites in a teleost (Chapter 2), the first pharmacological characterization of the rainbow trout gill β-AR (Chapter 3), and the first demonstration of a β_3-AR in hepatic

tissue (Chapter 4). However, several important questions remain unanswered. βARK and β-arrestin mRNA are known to be expressed in different rainbow trout tissues (Nickerson *et al.*, 2004). However, no study has yet to demonstrate βARK-mediated phosphorylation of a teleost β-AR. Nor has any study to date shown β-arrestin coupling to a teleost β-AR. The role of βARK and β-arrestin in the regulation of teleostean β-ARs definitely warrants further study. Pharmacology studies demonstrated the requirement to identify ligands that display subtype selectivity for the different β-ARs in teleosts in order to accurately characterize and examine *in vivo* regulation of a specific subtype. In addition, regulation of α_1-ARs in teleosts has never been studied. As α_1-ARs are known to be present in rainbow trout (Fabbri *et al.*, 1995a) and black bullhead (Fabbri *et al.*, 1995b) hepatic tissue, it is possible they play a reserve role to maintain AR signaling during stressor exposure. Studies to date on the ARs of teleosts have only just started to shed light on the complexity of this system. Future studies will require the use of both molecular and physiological tools in order to gain a more complete understanding of the teleost adrenergic system.

References

Abogadie, F.C., Bruch, R.C., and Farbman, A.I. (1995) G-protein subunits expressed in catfish olfactory receptor neurons. *Chem. Senses.* **20**:199-206.

Alquist, R.P. (1948) A study of adrenotropic receptors. *Am. J. Physiol.* **153**:586-600.

Auman, J.T., Siedler, F.J., and Slotkin, T.A. (2001a) Regulation of fetal cardiac and hepatic β-adrenoceptors and adenylyl cyclase signaling: terbutaline effects. *Am. J. Physiol.* **281**:R1079-R1089.

Auman, J.T., Siedler, F.J., Tate, C.A., and Slotkin, T.A. (2001b) β-Adrenoceptor-mediated signaling in the neonatal heart and liver: responses to terbutaline. *Am. J. Physiol.* **281**:R1895-R1901.

Barak, L.S., and Caron, M.G. (1995) Modelling of seqestration and down-regulation in cells containing beta$_2$-adrenergic receptors. *J. Recept. Sign. Transduc. Res.* **15(1-4)**:677-690.

Barnes, P.J. (1995) Beta-adrenergic receptors and their regulation. *Am. J. Crit. Care Med.* **152**:838-860.

Benovic, J.L., DeBlasi, A., Stone, W.C., Caron, M.G., and Lefkowitz, R.J. (1989) β-Adrenergic receptor kinase: Primary structure delineates a multigene family. *Science.* **246**: 235-240.

Bergmeyer, H.U. (Ed.) (1983) Methods of enzymatic analysis, 3rd ed. Verlag Chemie, Weinhem.

Birnbaumer, L., and Birnbaumer, M. (1995) Signal transduction by G proteins:1994 edition. *J. Rec. Sig. Trans. Res.* **15(1-4)**:213-252.

Blom, N., Gammeltoft., S., and Brunak, S. (1999) Sequence- and structure-based prediction of eukaryotic protein phosphorylation sites. *J. Mol. Biol.* **294**:1351-1362.

Bousquet-Mélou, A., Galitzky, J., Moreno, C.M., Berlan, M., Lafontan, M. (1995) Desensitization of β-adrenergic responses in adipocytes involves receptor subtypes and cAMP phosphodiesterase. *Eur. J. Pharmacol.* **289**:235-247.

Bouvier, M., Hausdorff, W. P., De Blasi, A., O'Dowd, B.F., Kobilka, B.K., Caron, M.G., and Lefkowitz, R.J. (1988) Removal of phosphorylation sites from the β_2-adrenergic receptor delays onset of agonist-promoted desensitization. *Nature.* **333**:370-373.

Brambilla, G., Bocca, A., Delise, M., and Guandalini, E. (1994) Residues of clenbuterol in tissues of the rainbow trout (*Oncorhynchus mykiss*). *Vet. Res. Comm.* **18**:37-42.

Brighenti, L., Puviani, A.C., Gavioli, M.E., Fabbri, E., and Ottolenghi, C. (1987a) Catecholamine effect on cyclic adenosine3':5'-monophosphate level in isolated catfish hepatocytes. *Gen. Comp. Endocrinol.* **68**: 216-223.

Brighenti, L., Puviani, A.C., Gavioli, M.E., and Ottolenghi, C. (1987b) Mechanisms involved in catecholamine effect on glycogenolysis in catfish isolated hepatocytes. *Gen. Comp. Endocrinol.* **66**: 306-313.

Brodde, O.E., and Michel, M.C. (1999) Adrenergic and muscarinic receptors in the human heart. *Pharmacol. Rev.* **51(4)**:651-689.

Brown, L., Deighton, N.M., Bals, S., Söhlmann, W., Zerkowski, H.R., Michel, M.C., and Brodde, O.E. (1992) Spare receptors for β-adrenoceptor-mediated positive inotropic effects of catecholamines in the human heart. *J. Cardiovasc. Pharmacol.* **19**:222-232.

Bylund, D.B. (1992) Subtypes of α_1- and α_2-adrenergic receptors. *FASEB J.* **6**:832-839.

Chu, A.C., Sindelar, D.K., Igawa, K., Sherck, S., Neal, D.W., Emshwiller, M., and Cherrington, A.D. (2000) The direct effects of catecholamines on hepatic glucose production occur via α_1- and β_2-receptors in the dog. *Am. J. Physiol.* **279**:E463-E473.

D'Allaire, F., Atgié, C., Mauriège, P., Simard, P.M., and Bukowiecki, L.J. (1995) Characterization of β_1 and β_3-adrenoceptors in intact brown adipocytes of the rat. *Brit. J. Pharmacol.* **114**:275-282.

Danulat, E., and Mommsen, T.P. (1990) Norepinephrine: a potent activator of glycogenolysis and gluconeogenesis in rockfish hepatocytes. *Gen. Comp. Endocrinol.* **78**:12-22.

Daveloose, D., Vezin, H., Basse, F., and Viret, J. (1993) Fluidity of chicken ventricular plasma membrane during development *in-ovo* and after birth: spin labelling and fluorescence studies. *J. Mol. Cell. Cardiol.* **25**:1439-1444.

Dugan, S.G., and Moon, T.W. (1998) Cortisol does not affect hepatic α- and β-adrenoceptor properties in rainbow trout (*Oncorhynchus mykiss*). *Fish Physiol. Biochem.* **18**:343-352.

Duman, R.S., and Nestler, E.J. (1995) Signal transduction pathways for catecholamine receptors. In *Psychopharmacology: The Fourth Generation of Progress.* (eds. Bloom, F.E., and Kupfer, D.J.) Chapter 27, Raven Press Ltd.

Dzimiri, N. (1999) Regulation of β-adrenoceptor signaling in cardiac function and disease. *Pharmacol. Rev.* **51(3)**:465-501.

Exton, J.H. (1985) Mechanisms involoved in α-adrenergic phenomena. *Am. J. Physiol.* **248**:E633-E647.

Fabbri, E., Brighenti, L., Ottolenghi, C., Puviani, A.C., and Capuzzo, A. (1992) β-Adrenergic receptors in catfish liver membranes: charaterization and coupling to adenylate cyclase. *Gen. Comp. Endocrinol.* **85**:254-260.

Fabbri, E., and Moon, T.W. (1994) Adrenergic receptors and second messenger systems in livers of vertebrates. In *Perspectives in Comparative Endocrinology*. Davey, K.G., Peter, R.E., and Tobe, S.S. eds. pp. 499-506. NRCC, Ottawa, Canada.

Fabbri, E., Capuzzo, A., Gambarotta, A., and Moon, T.W. (1995a) Characterization of adrenergic receptors and related transduction pathways in the liver of the rainbow trout. *Comp. Biochem. Physiol.* **112B**:643-651.

Fabbri, E., Gambarotta, A., and Moon, T.W. (1995b) Adrenergic signaling and second messenger production in hepatocytes of two fish species. *Gen. Comp. Endocrinol.* **99**:114-124.

Fabbri, E., Capuzzo, A., and Moon, T.W. (1998a) The role of circulating catecholamines in the regulation of fish metabolism: an overview. *Comp. Biochem. Physiol.* **120C**:177-192.

Fabbri, E., Barbin, L., Capuzzo, A., and Biondi, C. (1998b) Adenylyl cyclase activity and glucose release from the liver of the European eel, *Anguilla anguilla*. *Am. J. Physiol.* **275**:R1563-R1570.

Fabbri, E., Buzzi, M., Biondi, C., and Capuzzo, A. (1999) α-Adrenoceptor-mediated glucose release from perifused catfish hepatocytes. *Life Sci.* **65**:27-35.

Fabbri, E., Selva, C., Moon, T.W., and Capuzzo, A. (2001) Characterization of [^{3}H]CGP 12177 binding to β-adrenergic receptors in intact eel hepatocytes. *Gen. Comp. Endocrinol.* **121**:223-231.

Fabbri, E., Selva, C., Piano, A., Caselli, F., and Capuzzo, A. (2002) Identification and properties of a Gs protein in catfish liver membranes. *Gen. Comp. Endocrinol.* **125**:340-348.

Ferguson, S.G. (2001) Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol. Rev.* **53**:1-24.

Finkenbine, S.S., Gettys, T.W., and Burnett, K.G. (2002) Beta-adrenergic receptors on leukocytes of the channel catfish, *Ictalurus punctatus*. *Comp. Biochem. Physiol.* **131C**:27-37.

Fuchs, D.A., and Albers, C. (1988) Effects of adrenaline and blood gas conditions on red cell volume and intra-erythrocytic electrolytes in the carp, *Cyprinus carpio*. *J. Exp. Biol.* **137**:457-476

Galvez, F., Reid, S.D., Hawkings, G., and Goss, G.G. (2002). Isolation and characterization of mitochondria-rich cell types from the gill of freshwater rainbow trout. *Am. J. Physiol.* **282**:R658-R668.

Gamperl, A.K., Vijayan, M.M., and Boutilier, R.G. (1994a) Experimental control of stress hormone levels in fishes: techniques and applications. *Rev. Fish Biol. Fish.* **4**:215-255.

Gamperl, A.K., Wilkinson, M., and Boutilier, R.G. (1994b) β-Adrenoceptors in trout (*Oncorhynchus mykiss*) heart: characterization, quantification, and effects of repeated catecholamine exposure. *Gen. Comp. Endocrinol.* **95**:259-272.

Gamperl, A.K., Vijayan, M.M., Pereira, C., and Farrell, A.P. (1998) β-Receptors and stress protein 70 expression in hypoxic myocardium of rainbow trout and chinook salmon. *Am. J. Physiol.* **274**:R428-R436.

Garcìa-Sàinz, J.A., Cruz-Muñoz, M.E., and Romero-Avila, M.T. (1996) Characterization of the β_2-adrenoceptors of dog liver. *Comp. Biochem. Physiol.* **115C**:61-65.

Gilmour, K.M., Didyk, N.E., Reid, S.G., and Perry, S.F. (1994) Down-regulation of red blood cell β-adrenoceptors in response to chronic elevation of plasma catecholamine levels in the rainbow trout. *J. Exp. Biol.* **186**:309-314.

Granneman, J.G. (2001) The putative β_4-adrenergic receptor is a novel state of the β_1-adrenergic receptor. *Am. J. Physiol.* **43**:E199-E202.

Guibbolini, M., and Lahlou, B. (1987) Basal and stimulated adenylate cyclase activity in the gill epithelium of the rainbow trout. *Gen. Comp. Endocrinol.* **67**: 178-188.

Hadcock, J.R., and Malbon, C.C. (1988) Down-regulation of β-adrenergic receptors: Agonist-induced reduction in receptor mRNA levels. *Proc. Natl. Acad. Sci.* **85**:5021-5025.

Hadcock, J.R., Ros, M., and Malbon, C.C. (1989a) Agonist regulation of β-adrenergic receptor mRNA. *J. Biol. Chem.* **264**:13956-13961.

Hadcock, J.R., Wang, H., and Malbon, C.C. (1989b) Agonist induced destabilization of β-adrenergic receptor mRNA. *J. Biol. Chem.* **264**:19928-19933.

Harding, S.E., Brown, L.A., Wynne, D.G., Davies, C.H., and Poole-Wilson, P.A. (1994) Mechanisms of β adrenoceptor desensitization in the failing human heart. *Cardiovasc. Res.* **28(10)**:1451-1460.

Hein, L., and Kobilka, B.K. (1995) Adrenergic receptor signal transduction and regulation. *Neuropharmacol.* **34(4)**:357-366.

Hernández-Sotomayor, S.M.T., Macías-Silva, M., Plebański, M., and García-Sáinz, J.A. (1988) Homologous and heterologous β-adrenergic desensitization in hepatocytes. Additivity and effect of pertussis toxin. *Biochim. Biophys. Acta.* **972**:311-319.

Jahns, R., Borgeses, F., Lindenthal, S., Straub, A., Motais, R., and Fiévet, B. (1996) Trout red blood cell arrestin (TRCarr), a novel member of the arrestin family: cloning, immunoprecipitation and expression of recombinant TRCarr. *Biochem. J.* **316**:497-506.

Janssens, P.A., and Grigg, J.A. (1988) Binding of adrenergic ligands to liver plasma membrane preparations from the axolotl, *Ambystoma mexicanum*; the toad, *Xenopus laevis*; and the Australian lungfish, *Neoceratodus forsteri*. *Gen. Comp. Endocrinol.* **71**:524-530.

Johnson, M. (1998) The β-adrenoceptor. *Am. J. Respir. Crit. Care Med.* **158**:S146-S153.

Jozefowski, S.J., and Plytycz, B. (1998) Characterization of β-adrenergic receptors in fish and amphibian lymphoid organs. *Dev. Comp. Immunol.* **22(5/6)**:587-603.

Kaumann, A.J. (1997) Four β-adrenoceptor subtypes in the mammalian heart. *TiPS.* **18**:70-76.

Kawai, Y., Powell, A., and Arinze, I. (1986) Adrenergic receptors in human liver plasma membranes: predominance of β_2- and α_1-receptor subtypes. *J. Clin. Endocrinol. Metab.* **62**:827-832.

Keen, J.E., Vianzon, D.M., Farrell, A.P., and Tibbits, G.F. (1993) Thermal acclimation alters both adrenergic sensitivity and adrenoceptor density in cardiac tissue of rainbow trout. *J. Exp. Biol.* **181**:27-47.

Lands, A.M., Arnold, A., McAuliff, J.P., Luduena, F.P., and Brown, T.G. (1967) Differentiation of receptor systems activated by sypathomimetic amines. *Nature.* **215**:597-598.

Lefkowitz, R.J. (1978) Identification and regulation of alpha- and beta-adrenergic receptors. *Fed. Proc.* **193**:123-129.

Lefkowitz, R.J., and Caron, M.G. (1986) Regulation of adrenergic receptor function by phosphorylation. *J. Mol. Cell. Cardiol.* **18**:885-895.

Lefkowitz, R.J. (2000) The superfamily of heptahelical receptors. *Nature Cell Biol.* **2**:E133-E136.

Liang, W., and Mills, S. (2001) Profile of ligand binding to the porcine beta2-adrenergic receptor. *J. Anim. Sci.* **79**:877-883.

Lima, J.J. (1996) Relationship between β adrenoceptor occupancy and receptor down-regulation induced by β antagonists with intrinsic sympathomimetic activity. *J. Recept. Sign. Transduc. Res.* **16(5&6)**:357-372.

Lohse, M.J., Benovic, J.L., Codina, J., Caron, M.G., and Lefkowitz, R.J. (1990) β-Arrestin: A protein that regulates β-adrenergic receptor function. *Science*. **248**:1547-1550.

Lohse, M.J. (1993) Molecular mechanisms of membrane receptor desensitization. *Biochim. Biophys. Acta*. **1179**:171-188.

Lohse, M.J., Krasel, C., Winstel, R., and Mayor Jr, F. (1996) G-protein-coupled receptor kinases. *Kidney Intl.* **49**:1047-1052.

Lomasney, J.W., Allen, L.F., and Lefkowitz, R.J. (1995) Cloning and regulation of catecholamine receptor genes. In *Molecular Endocrinology: Basic Concepts and Clinical Correlations*. (ed. Weintraub, B.D.) Chapter 10, Raven Press Ltd. New York.

Lortie, M.B., and Moon, T.W. (2003) The rainbow trout skeletal muscle β-adrenergic system: characterization and signaling. *Am. J. Physiol.* **284**:R689-R697.

Lortie, M.B., Arnason, T., Dugan, S.G., Nickerson, J.G., and Moon, T.W. (2004) The impact of feeding β_2-adrenergic agonists on rainbow trout muscle β_2-adrenoceptors and protein synthesis. *J. Fish Biol.* In Press.

Marshall, W.S. (2002) Na^+, Cl^-, Ca^{2+}, and Zn^{2+} transport by fish gills: retrospective review and prospective synthesis. *J. Exp. Zool.* **293**:264-283.

Marullo, S., Nantel, F., Strosberg, A.D., and Bouvier, M. (1995) Variability in the regulation of β-adrenoceptor subtypes. *Biochem. Soc. Trans.* **23**:126-129.

Manzl, C., Schubert, M., Schwarzbaum, P.J., and Krumschnabel, G. (2002) Effects of chemical anoxia on adrenergic responses of goldfish hepatocytes and the contribution of α- and β-adrenoceptors. *J. Exp. Zool.* **292(5)**:468-476.

McDonald, P.H., and Lefkowitz, R.J. (2001) β-arrestins: new roles in regulating heptahelical receptors' functions. *Cell Signal.* **13**:683-689.

McKendry, J.E., and Perry, S.F. (2001) Cardiovascular effects of hypercarbia in rainbow trout (*Onchorhynchus mykiss*): a role for externally oriented chemoreceptors. *J. Exp. Biol.* **204**:115-125.

McNeel, R.L., and Mersmann, H.J. (1999) Distribution and quantification of beta1-, beta2-, and beta3-adrenergic receptor subtype transcripts in porcine tissues. *J. Anim. Sci.* **77(3)**:611-621.

Medina-Martínez, O., and García-Sáinz, J.A. (1993) Hepatocyte homologous β_2-adrenergic desensitization is associated with a decrease in number plasma membrane β_2-adrenoceptors. *Eur. J. Pharmacol.* **244**:145-151.

Mendelsohn, S.A., Cherksey, B.D., and Degnan, K.J. (1981) Adrenergic regulation of chloride cell secretion across the opercular epithelium: the role of cAMP. *J. Comp. Physiol.* **145**:29-35.

Milligan, G., Svoboda, P., and Brown, C.M. (1994) Why are there so many adrenoceptor subtypes? *Biochem. Pharmacol.* **48(6)**:1059-1071.

Mills, S. (2001) Biological basis of the ractopamine response. *Ractopamine at One Year of Commercial Application.* International Animal Agriculture and Food Science Conference. Indianapolis, IN. (http://www.fass.org/fass01/pdfs/Mills.pdf)

Mills, S.E., (2002) Implications of feedback regulation of β-adrenergic signaling. *J. Anim. Sci.* **80**:E30-E35.

Mills, S.E., Spurlock, M.E., and Smith, D.J. (2003a) β-Adrenergic receptor subtypes that mediate ractopamine stimulation of lipolysis. *J. Anim. Sci.* **81**:662-668.

Mills, S.E., Kissel, J., Bidwell, C.A., and Smith, D.J., (2003b) Stereoselectivity of porcine β-adrenergic receptors for ractopamine stereoisomers. *J. Anim. Sci.* **81**:122-129.

Mommsen, T.P., Moon, T.W., Walsh, P.J. (1994) Hepatocytes: isolation, maintenance, and utilization. In: Biochemistry and molecular biology of fishes, vol 3. Hochacka and Mommsen (eds.). pp. 355-373.

Moon, T.W., and Mommsen, T.P. (1990) Vasoactive peptides and phenylephrine actions in isolated teleost hepatocytes. *Am. J. Physiol.* **259**:E644-E649.

Moon, T.W., Busby, E.R., Cooper, G.A., and Mommsen, T.P. (1999) Fish hepatocyte glycogen phosphorylase – a sensitive indicator for hormonal modulation. *Fish Physiol. Biochem.* **21**:15-24.

Morgan, I.J., and Potts, W.T.W. (1995) The effects of the adrenoreceptor agonists phenylephrine and isoproterenol on the intracellular ion concentrations of branchial epithelial cells of brown trout (*Salo trutta* L.). *J. Comp. Physiol. B.* **165**:458-463.

Munson, P.F., and Rodbard, D. (1980) LIGAND: a versatile computerized approach for characterization of ligand binding system. *Anal. Biochem.* **107**:220-239.

Nekvasil, N.P. and Olson, K.R. (1985) Localization of ^{3}H-norepinephrine binding sites in the trout gill. *J. Exp. Zool.* **235**:309-313.

Nickerson, J.G., Dugan, S.G., Drouin, G., and Moon, T.W. (2001) A putative β_2-adrenoceptor from the rainbow trout (*Oncorhynchus mykiss*): molecular characterization and pharmacology. *Eur. J. Biochem.* **268**:6465-6472.

Nickerson, J.G., Dugan, S.G., Drouin, G., Perry, S.F., and Moon, T.W. (2003) Activity of the unique β-adrenergic Na^+/H^+ exchanger in trout erythrocytes is controlled by a novel β_3-AR subtype. *Am. J. Physiol.* **285**:R526-R535.

Nickerson, J.G., Drouin, G., Perry, S.F., and Moon, T.W. (2004) *In vitro* regulation of β-adrenoceptor signaling in the rainbow trout, *Oncorhynchus mykiss*. *Fish Physiol. Biochem.* In Press.

Olson, K.R. (2002) Vascular anatomy of the fish gill. *J. Exp. Zool.* **293**:214-231.

Olsson, H.I., Yee, N., Shiels, H.A., Brauner, C., and Farrell, A.P. (2000) A comparison of myocardial β-adrenoceptor density and ligand binding affinity among selected teleost fishes. *J. Comp. Physiol. B.* **170**:545-550.

Ordway, G.A., O'Donnell, J.M., and Frazer, A., (1987) Effects of clenbuterol on central *beta*-1 and *beta*-2 adrenergic receptors of the rat. *J. Pharmacol. Exp. Therap.* **241**:187-195.

Ottolenghi, C., Fabbri, E., Puviani, A.C., Gavioli, M.E., and Brighenti, L. (1988) Adenylate cyclase of catfish hepatocyte membranes: basal properties and sensitivity to catecholamines and glucagon. *Molec. Cell. Endocrinol.* **60**:163-168.

Payan, P., and Girard, J.P. (1977) Adrenergic receptors regulating patterns of blood flow through the gills of trout. *Am. J. Physiol.* **232(1)**:H18-H23.

Perry, S.F., Payan, P., and Girard, J.P. (1984) Adrenergic control of branchial chloride transport in the isolated perfused head of the freshwater trout (*Salmo gairdneri*). *J. Comp. Physiol. B.* **154**:269-274.

Perry, S.F., and Reid, S.D. (1992) The relationship between β-adrenoceptors and adrenergic responsiveness in trout (*Oncorhynchus mykiss*) and eel (*Anguilla rostrata*) erythrocytes. *J. Exp. Biol.* **167**:235-290.

Perry, S.F., and Reid, S.D. (1993) β-Adrenergic signal transduction in fish: interactive effects of catecholamines and cortisol. *Fish Physiol. Biochem.* **11(1-6)**:195-203.

Perry, S.F., Reid, S.G., and Salama, A. (1996) The effects of repeated physical stress on the β-adrenergic response of the rainbow trout red blood cell. *J. Exp. Biol.* **199**:549-562.

Perry, S.F. (1998) Relationships between branchial chloride cells and gas transfer in freshwater fish. *Comp. Biochem. Physiol.* **119A**:9-16.

Perry, S.F., and Reid, S.G. (2002) Cardiorespiratory adjustments during hypercarbia in rainbow trout *Oncorhynchus mykiss* are initiated by external CO_2 receptors in the first gill arch. *J. Exp. Biol.* **205**:3357-3365.

Randall, D.J., and Perry, S.F. (1992) Catecholamines. In *Fish Physiology* Vol XIIB. (eds. Randall, D.J., and Hoar, W.S) pp. 255-300. Academic Press, N.Y., USA.

Rao, G.S., Seidler, F.J., and Slotkin, T.A. (1995) Modulation of hepatic adrenergic receptor ontogeny by thyroid hormone. *Res. Comm. Molec. Pathol. Pharmacol.* **87(1)**:27-42.

Raymond, J.R. (1995) Multiple mechanisms of receptor-G protein signaling specificity. *Am. J. Physiol.* **269**:F141-F158.

Reid, S.D., Moon, T.W., and Perry, S.F. (1992) Rainbow trout hepatocytes β-adrenoceptors, catecholamine responsiveness, and effects of cortisol. *Am. J. Physiol.* **262**:R794-R799.

Reid, S.D., Lebras, Y, and Perry, S.F. (1993) The *in vitro* effect of hypoxia on the trout erythrocyte β-adrenergic signal transduction system. *J. Exp. Biol.* **176**:103-116.

Reid, S.G., Furimsky, M., and Perry, S.F., (1994) The effects of repeated physical stress or fasting on catecholamine storage and release in the rainbow trout, *Oncorhynchus mykiss*. *J. Fish Biol.* **45**:365-378.

Reid, S.G., and Perry, S.F. (1995) The effects of hypoxia, *in vivo*, on red blood cell β-adrenoceptors in the rainbow trout, *Oncorhynchus mykiss*. *Fish Physiol. Biochem.* **14**:519-524.

Reid, S.G., Bernier, N.J., and Perry, S.F. (1998) The adrenergic stress response in fish: control of catecholamine storage and release. *Comp. Biochem. Physiol.* **120C**:1-27.

Ruffolo Jr., R.R., Nichols, A.J., Stadel, J.M., and Hieble, J.P. (1991) Structure and function of α-adrenoceptors. *Pharmacol. Rev.* **43(4)**:475-505.

Sarsero, D., Molenaar, P., Kaumann, A.J., and Freestone, N.S. (1999) Putative β_4-adrenoceptors in rat ventricle mediate increases in contractile force and cell Ca^{2+}: comparison with atrial receptors and relationship to (-)-[^{3}H]-CGP 12177 binding. *Brit. J. Pharmacol.* **128**:1445-1460.

Shapell, N.W., Feil, V.J., Smith, D.J., Larsen, G.L., and McFarland, D.C. (2000) Response of C_2C_{12} mouse and turkey skeletal muscle cells to the β-adrenergic agonist ractopamine. *J. Anim. Sci.* **78**:699-708.

Sillence, M.N., Matthews, M.L., Moore, N.G., and Reich, M.M. (1995) Effects of BRL-47672 on growth, β_2-adrenoceptors, and adenylyl cyclase activation in female rats. *Am. J. Physiol.* **268**:E159-E167.

Steevens, J.A., Baumann, P.C., and Jones, S.B. (1996) A comparison of β-adrenoceptors and muscarinic cholinergic receptors in tissues of brown bullhead catfish (*Ameiurus nebulosus*) from the Black River and Old Woman Creek, Ohio. *Environ. Toxicol. Chem.* **15(9)**:1551-1554.

Strader, C.D., Sigal, I.S., and Dixon, R.A.F. (1989) Structural basis of β-adrenergic receptor function. *FASEB J.* **3**:1825-1832.

Strosberg, A.D. (1993) Structure, function, and regulation of adrenergic receptors. *Prot. Sci.* **2**:1198-1209.

Strosberg, A.D. (1997) Structure and function of the β_3-adrenergic receptor. *Annu. Rev. Pharmacol. Toxicol.* **37**:421-450.

Sulakhe, S.J., Pulga, V.B., and Tran, S. (1988) Hepatic α_1 and β adrenergic receptors in various animal species. *Molec. Cell. Biochem.* **83**:81-88.

Summers, R.J., and McMartin, L.R. (1993) Adrenoceptors and their second messenger systems. *J. Neurochem.* **60**:10-23.

Sundin, L.I. (1995) Responses of the branchial circulation to hypoxia in the Atlantic cod, *Gadus morhua*. *Am. J. Physiol.* **268(3)**:R771-R778.

Tetens, V., Lykkeboe, G., and Christensen, N.J. (1988) Potency of adrenaline and noradrenaline for β-adrenergic proton extrusion from red cells of rainbow trout, *Salmo gairdneri*. *J. Exp. Biol.* **134**:267-280.

Thomas, S., Kinkead, R., Walsh, P.J., Wood, C.M., and Perry, S.F. (1991) Desensitization of adrenaline-induced red blood cell H^+ extrusion *in vitro* after chronic exposure of rainbow trout to moderate environmental hypoxia. *J. Exp. Biol.* **156**:233-248.

Thompson, J.D., Higgins, D.G., and Gibson T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**:4673-4680.

van den Thillart, G., Vianen, G., Ponce, M.C., Lelieveld, H., Nieveen, M., Van Raaij, M., Steffens, A., and Zaagsma, J. (2001) Differential role of adrenoceptors in control of plasma glucose and fatty acids in carp, *Cyprinus carpio*. *Am. J. Physiol.* **281(2)**:R615-R624.

Van Raaij, M., van den Thillart, G., Pit, D., Balm, P., and Steffens, A. (1996) Substrate-mobilization and hormonal changes in rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*) during deep hypoxia and subsequent recovery. *J. Comp. Physiol. B.* **166**:443-452.

Vandenberg, G.W., and Moccia, R.D., (1998) Growth performance and carcass composition of rainbow trout, *Oncorhynchus mykiss* (Walbaum), fed the β-agonist ractopamine. *Aquacul. Res.* **29**:469-479.

Vianen, G.J., Obels, P.P. van den Thillart, G.E., and Zaagsma, J. (2002) Beta-adrenoceptors mediate inhibition of lipolysis in adipocytes of tilapia (*Oreochromis mossambicus*). *Am. J. Physiol.* **282(2)**:E318-E325.

Vos, P., Davenport, P.A., Artymyshyn, R.P., Frazer, A., and Wolfe, B.B., (1987) Selective regulation of beta-2 adrenergic receptors by the chronic administration of the lipophilic beta adrenergic receptor agonist clenbuterol: an autoradiographic study. *J. Pharmacol. Exptl. Therap.* **242**:707-712.

Wallukat, G. (2002) The β-adrenergic receptors. *Herz.* **27**:683-690.

Wendelaar Bonga, S.E. (1997) The stress response in fish. *Physiol. Rev.* **77(3)**:591-625.

Wilson, J.M., and Laurent P. (2002) Fish gill morphology: inside out. *J. Exp. Zool.* **293**:192-213.

Wright, P.A., Perry, S.F., and Moon, T.W. (1989) Regulation of hepatic gluconeogenesis and glycogenolysis by catecholamines in rainbow trout during environmental hypoxia. *J. Exp. Biol.* **147**:169-188.

Wolfe, B.B., Harden, T.K., and Molinoff, P.B. (1976) β-Adrenergic receptors in rat liver: effects of adrenalectomy. *Proc. Natl. Acad. Sci. USA.* **73**:1343-1347.

Wolfe, J.T., Wang, H., Howard, J., Garrison J.C., and Barett, P.Q. (2003) T-type calcium regulation by specific G-protein βγ subunits. *Nature.* **424**:209-213.

Zhao, J., Cannon, B., and Nedergaard, J. (1998) Thermogenesis is β_3- but not β_1-adrenergically mediated in rat brown fat cells, even after cold acclimation. *Am. J. Physiol.* **275**:R2002-R2011.

Zhong, H., and Minneman, K.P. (1999) α_1-Adrenoceptor types. *Eur. J. Pharmacol.* **375**:261-276.

APPENDIX

In addition to my own thesis work, I collaborated on two papers of Jamie Nickerson. My part in each was to pharmacologically characterize a trout β-AR subtype. The abstract for each paper is presented here. The figure for the characterization of the trout β_2-AR for Nickerson *et al.* (2001) was modified and presented in Chapter 2 (Fig. 2.1). The figures for the characterization of the red blood cell β_{3b}-AR for Nickerson *et al.* (2003) were modified and are shown here (Figs. A1 and A2). The clenbuterol data presented in Chapter 2 was part of a larger collaborative project with Michel Lortie. In this study, I assisted in the feeding of the fish, tissue collection and binding assays. The abstract of the paper (Lortie *et al.*, 2004) for the other part of the clenbuterol study is also presented here.

A Putative β_2-Adrenoceptor from the Rainbow Trout (*Oncorhynuchus mykiss*): Molecular Characterization and Pharmacology.

James G. Nickerson, Stephen G. Dugan, Guy Drouin and Thomas W. Moon.

2001. *Eur. J. Biochem.* **268:**6465-6472.

Extensive molecular characterization of mammalian β-adrenoceptors has revealed complex modes of regulation and interaction. Relatively little attention, however, has focused on adrenoceptors from early branching vertebrates such as fish. Using an RT-PCR approach we have cloned a rainbow trout β_2-adrenoceptor (RbT β_2-AR) gene which codes for a 409 amino acid protein with the same seven transmembrane domain structure as its mammalian counterparts. This RbT β_2-AR shares a high degree of amino acid sequence conservation with other vertebrate β_2-ARs. The conclusion that this sequence is a RbT β_2-AR is further supported by phylogenetic analysis of vertebrate β-AR sequences and competitive pharmacological binding data. RNase protection assays demonstrate that the RbT β_2-AR gene is highly expressed in the liver, red muscle, and white muscle with lower levels of expression in the gills, heart, kidney and spleen of the rainbow trout. The lack of regulatory phosphorylation sites within the G-protein binding domain of the RbT β_2-AR sequence suggests that the *in vivo* control of trout β_2-AR signaling differs substantially from that of mammals.

Activity of the unique ß-adrenergic Na^+/H^+ exchanger in trout erythrocytes is controlled by a novel β_3-AR subtype

James G. Nickerson, Stephen G. Dugan, Guy Drouin, Steve F. Perry, and Thomas W. Moon 2003. *Am. J. Physiol.* **285:**R526-R535.

β-Adrenoceptors (β-ARs) are seven transmembrane domain, G-protein coupled receptors that transduce the cellular effects of epinephrine and norepinephrine and play a pivotal role in the vertebrate stress response. This study reports the cloning and characterization of two previously unreported β-ARs from the rainbow trout (*Oncorhynchus mykiss*). Phylogenetic analysis of amino acid sequences indicates that both β-ARs are homologs of the mammalian β_3-AR. Analysis of tissue expression patterns indicates that one of these trout β_3-adrenoceptors (β_{3a}-AR) is highly expressed in gill and heart while the second (β_{3b}-AR) is highly expressed by red blood cells (RBC). Expression of the β_{3b}-AR in the red cell coupled with the finding of a single category of β-AR binding sites on red cell membranes provides strong evidence for the control of the trout red cell β-AR Na^+/H^+ exchanger (βNHE) activity by signaling through this β_{3b}-subtype and not through a β_1-subtype as previously proposed. The red cell specific trout β_{3b}-AR exhibits binding characteristics that distinguish this receptor from each of the three pharmacologically defined categories of mammalian β-ARs (β_1-, β_2-, and β_3-AR). This study is the first to report the presence of a β_3-AR subtype in a fish species and the proposal that the β_{3b}-AR controls red blood cell βNHE activity represents a novel role for the β_3-AR subtype in vertebrates.

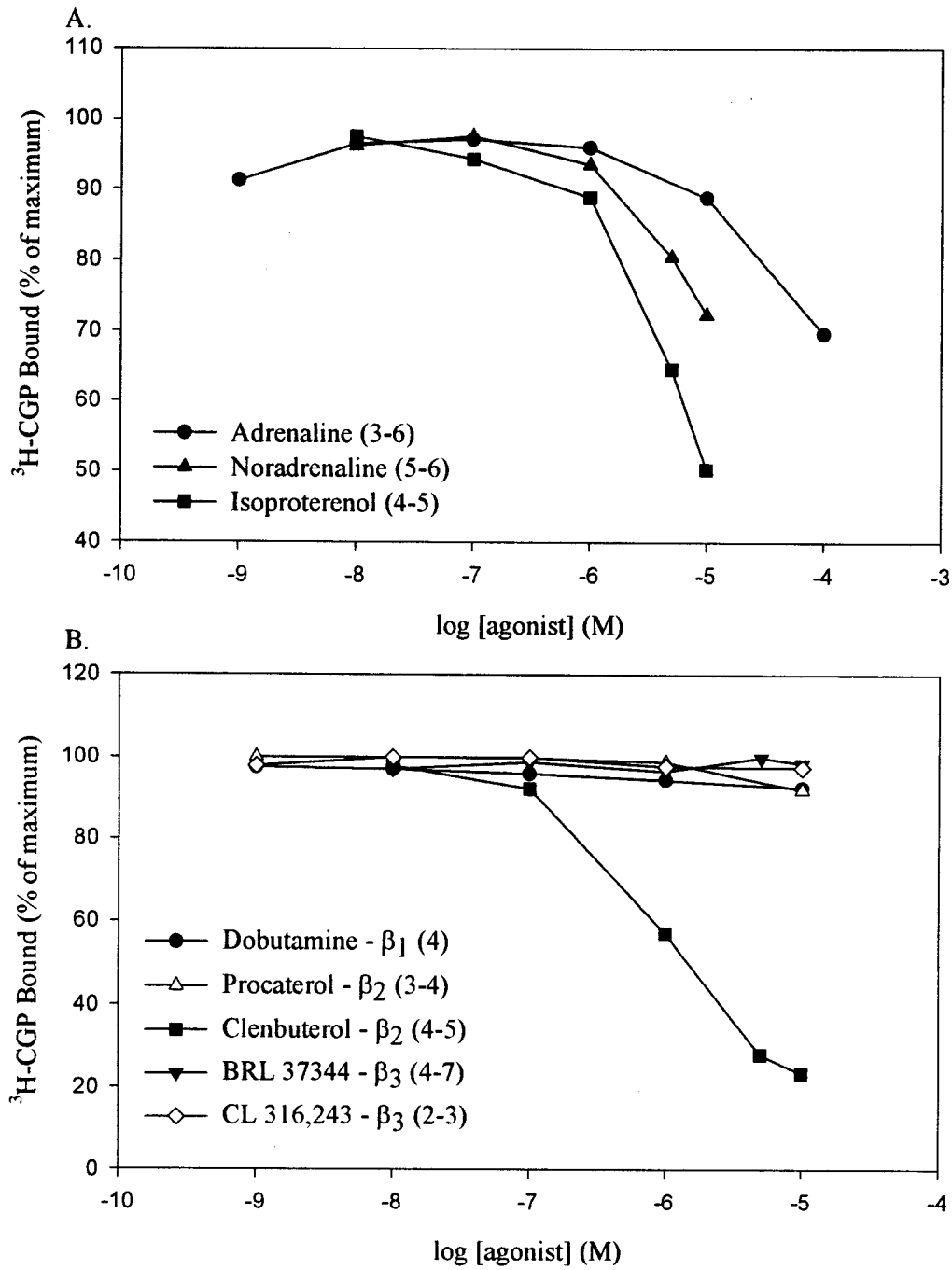


Figure A1. Competitive displacement of ^{3}H-CGP in rainbow trout red blood cells by non-selective (A.) and selective (B.) agonists. Blood was sampled from the dorsal aorta and diluted 10X with saline Hanks'. Red blood cells were incubated for 45 min with a saturating concentration of ^{3}H-CGP (5 nM) alone or with increasing concentrations of the non-labeled agonists. Figure modified from Nickerson *et al.* (2003). Values represent means with sample size in brackets. Variation (SEM) for each data point was less than 5% and is omitted for clarity.

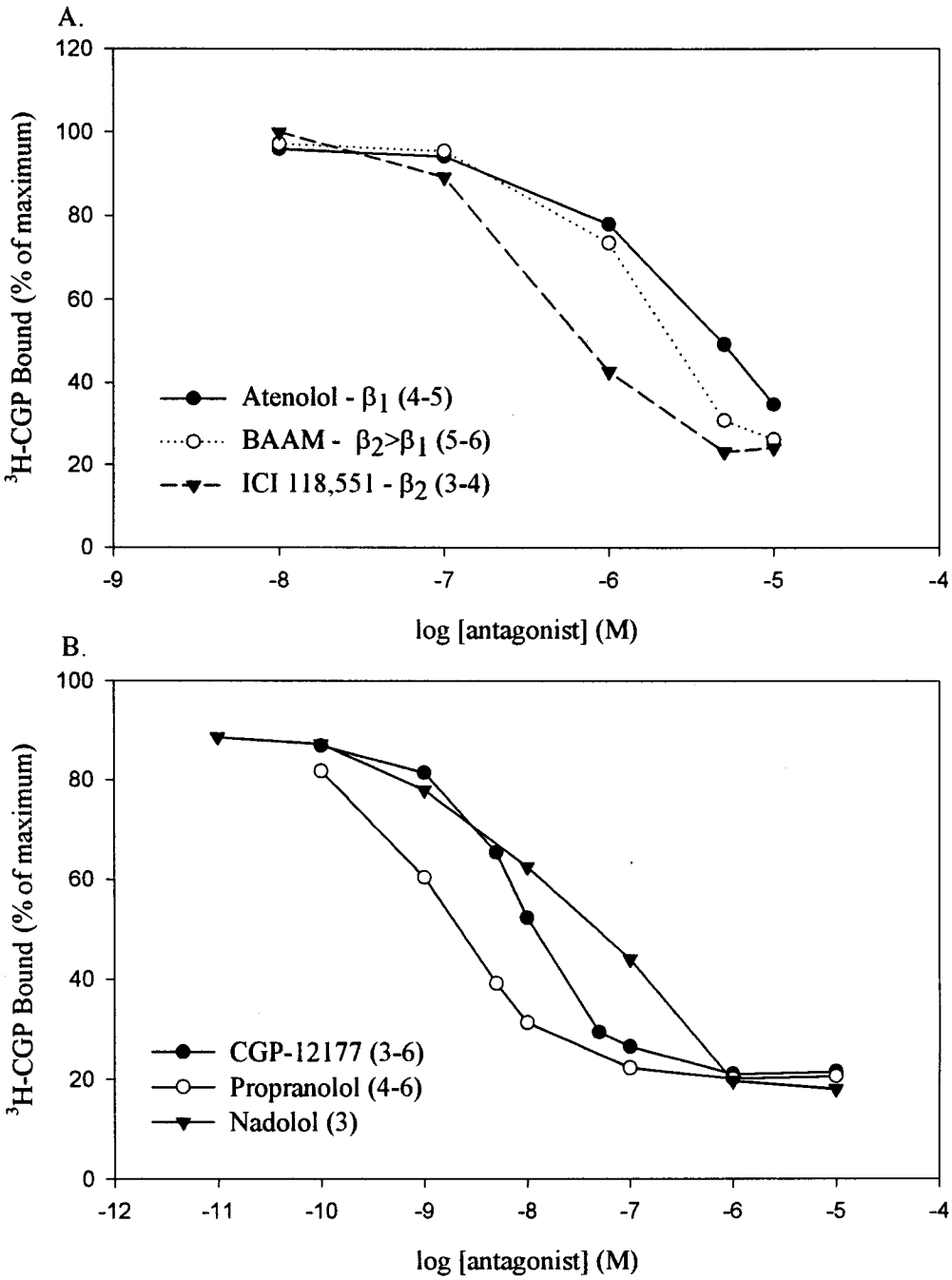


Figure A2. Competitive displacement of ^{3}H-CGP in rainbow trout red blood cells by selective (A.) and non-selective (B.) antagonists. Blood was sampled from the dorsal aorta and diluted 10X with saline Hanks'. Red blood cells were incubated for 45 min with a saturating concentration of ^{3}H-CGP (5 nM) alone or with increasing concentrations of the non-labeled antagonists. Figure modified from Nickerson *et al.* (2003). Values represent means with sample size in brackets. Variation (SEM) for each data point was less than 5% and is omitted for clarity.

The Impact of Feeding β_2-Adrenergic Agonists on Rainbow Trout Muscle β_2-Adrenoceptors and Protein Synthesis

Michel B. Lortie, Thomas Arnason, Stephen G. Dugan, James G. Nickerson and Thomas W. Moon

J. Fish Biol. 2004. In press.

The β_2-adrenergic agonists (β_2-AAs) clenbuterol (CLEN) and ractopamine (RACT) were fed to rainbow trout for 30-37 days and red and white muscle β_2-adrenoceptor (β_2-AR) binding characteristics and mRNA expression were assessed in parallel with fractional protein synthetic rates. Feeding CLEN or RACT had no significant effect on any body or physiological parameters measured. There were no significant differences in the number of binding sites (B_{max}) while a significant increase in the K_d was observed for the β-ARs of red and white muscle membranes isolated from β_2-AA-fed trout. No change in β_2-AR mRNA levels was observed with β_2-AA feeding, implying that these β_2-AAs do not act at the transcriptional level at least for the β_2-AR. However, β_2-AA treatments did significantly increase red and white muscle fractional protein synthesis rates in whole protein, myofibrillar protein and sarcoplasmic soluble protein fractions. Although not conclusive, this study supports a direct link between rainbow trout muscle β_2-ARs and muscle protein synthesis.