The rainbow trout skeletal muscle β-adrenergic system: characterization and signaling

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Lortie, Michel B., and Thomas W. Moon. The rainbow trout skeletal muscle β-adrenergic system: characterization and signaling. Am J Physiol Regul Integr Comp Physiol 284: R689–R697, 2003. First published November 21, 2002; 10.1152/ajpregu.00512.2002.—The presence and functionality of β-adrenoceptors (β-ARs) were examined in red (RM) and white muscle (WM) membranes isolated from the rainbow trout Oncorhynchus mykiss. Specific binding assays revealed the presence of a single class of binding sites with similar affinities in both muscle types (Kd in nM: 0.14 ± 0.03 and 0.18 ± 0.03 for RM and WM, respectively) but with a significantly higher number of binding sites in RM compared with WM (Bmax in fmol/mg protein: 3.22 ± 0.13 and 2.60 ± 0.13, respectively). Selective and nonselective β-adrenergic agonists (β-AAs) and antagonists indicated an atypical β-AR pharmacology. This result may represent a nonmammalian β-AR classification or, more likely, the presence of more than one β-AR subtype in trout muscles with similar affinities that could not be kinetically resolved. Adenyl cyclase (ACase) assays showed a dose-dependent increase in cAMP production as concentrations of β2-AAs increased in both muscle membranes with significantly higher basal cAMP production in RM compared with WM (cAMP production in pmol/mg protein: 24.67 ± 3.06 and 9.64 ± 3.45, respectively). The agonist-induced increase in cAMP production was blocked by the β-adrenergic antagonist propranolol, while the ACase activator forskolin increased cAMP production by 7- to 14-fold above basal and ~3-fold above all β-AAs tested. This study demonstrated the presence of atypical β2-ARs on RM and WM membranes of trout, suggesting that β2-AAs may be a tool to enhance protein accretion through this signaling pathway.

β-adrenoceptor; adenyl cyclase; adenosine 3',5'-cyclic monophosphate; Oncorhynchus mykiss

The adrenergic system is key to integrating and modulating many aspects of vertebrate, including fish, metabolism (10). The endogenous circulating catecholamine (CA) hormones epinephrine (Epi) and norepinephrine (NE) exert effects on target cells or tissues by binding to specific hormone receptors called adrenoceptors (ARs) that in turn activate intracellular transduction pathways. Both α- and β-ARs are found on fish and mammalian cells, and the pathways involved in the signal transduction system of fish ARs from the binding of CAs to α- and β-ARs to the ultimate effects of specific enzyme phosphorylation have been studied in isolated tissues, especially hepatocytes (10). Studies on the effects of CAs and the distribution of AR types in other metabolically important fish tissues, however, have received little or no attention. Of all the tissues in fish, skeletal muscle comprises the largest single tissue compartment, representing >50% of total body mass, a larger component when compared with other vertebrates (18). Indeed, there are more total insulin and insulin-like growth factor-I (IGF-I) receptors in skeletal muscle than liver of fish (30).

Pharmacological and molecular studies confirmed the presence of β1-, β2-, and β3-AR subtypes in rat white (glycolytic type II) and red (oxidative type I) skeletal muscles (7, 19, 21, 22, 37). β2-Adrenergic agonist (β2-AA) binding increased intracellular cAMP concentrations, primarily through the β2-AR subtype with slight to no cAMP increase observed using β1- and β3-AR agonists, respectively (37). Subsequent changes in cAMP, protein phosphorylation (by protein kinase A) and activation of cAMP-responsive elements (CRE) by cAMP response element binding protein (CREB) are believed to affect protein turnover by a transient stimulation of protein synthesis and a longer-lasting reduction in protein degradation (2, 3, 20, 23–25, 31, 34, 46). However, a mechanistic gap persists between phosphorylation of proteins or activation of CRE by cAMP and subsequent changes in protein turnover (2, 3, 24, 25, 27).

Recent molecular studies demonstrated the presence of a putative β2-AR from rainbow trout (Oncorhynchus mykiss) that shared a high degree of amino acid sequence conservation with other vertebrate β2-ARs. This AR type was highly expressed in liver, red muscle (RM), and white muscle (WM), but less so in gill, spleen, and kidney with no expression detected in red blood cells (32). No pharmacological characterization of β-ARs in teleost skeletal muscle is reported or its subsequent coupling to second messengers.

This study tested the hypothesis that rainbow trout RM and WM possess β2-ARs, and on β2-AA binding, a functional transduction cascade increased production of the second messenger cAMP. The main objectives of this study were to 1) demonstrate the presence of...
β-ARs in RM and WM of the rainbow trout, 2) pharmacologically characterize the subtype(s) of β-AR, and 3) demonstrate the causative association between β2-AA binding and activation of the subsequent transduction pathway.

METHODS

All animal protocols were reviewed and approved by the University of Ottawa Animal Care Protocol Review Committee and conform with the American Physiological Society “Guiding Principles for Research Involving Animals and Human Beings” (1).

Experimental animals. Female rainbow trout (Oncorhynchus mykiss), weighing approximately 125–200 g, were obtained from Linwood Acres Trout Farm (Campbellcroft, ON, Canada). Fish were transported to the University of Ottawa Aquatic Care Facility and were maintained in fiberglass holding tanks (1,275 liters) of well-aerated, dechloraminated City of Ottawa tap water at 13.0 ± 1.0°C. Fish were subjected to a constant 12:12-h light-dark photoperiod and fed five times per week with commercial trout pellets (Martin Mills 5 Aquatic Care Facility and underlying epaxial WM

All experiments used vitamin E (minimum), and 2,100 IU/kg vitamin D (minimum), 80 IU/kg (maximum), 1.0% calcium (actual), 0.85% phosphorus (minimum), 2.100 IU/kg vitamin D (minimum), 80 IU/kg vitamin E (minimum), and 200 IU/kg vitamin C (minimum)]. All experiments used fish obtained in September; receptor characterization experiments were conducted between October and December, and adenylyl cyclase (ACase) experiments were performed in January and February.

Muscle membrane preparation. Rainbow trout were netted and killed by a sharp blow to the head. The skin was removed, and the superficial RM and underlying epaxial WM were quickly and carefully excised, freeze clamped between aluminum blocks cooled in liquid N2, and stored at −80°C until membranes were prepared within 1 wk. Muscle membranes were isolated using a modification of methods previously validated for the rat (17, 21, 36, 39, 42) and the guinea pig (7). The muscle tissue samples were crushed to a fine powder using a porcelain mortar and pestle kept at liquid N2 temperatures. The powder was weighed and suspended in 5 vol of ice-cold basic Hanks’ medium (in mM: 136.9 NaCl, 5.4 KCl, 0.8 MgSO4, 7H2O, 0.33 Na2HPO4·7H2O, 0.44 KH2PO4, 5.0 HEPES, 5.0 HEPES-Na, 1.0 NaHCO3, and 0.43 PMSF) adjusted to pH 7.63. All subsequent procedures were carried out on ice (4°C), unless specified otherwise. The muscle was homogenized with six strokes (~10 s/stroke) of a Potter-Elvehjem Teflon-glass homogenizer attached to a commercial drill (Black and Decker) running at low speed. The resulting homogenate was centrifuged at 400 g for 10 min in a Sorvall RC 5B Plus (SS 34 rotor) at 4°C. The supernatant was filtered through nitex nylon mesh (250 μm; Sefar America, Kansas City, MO); the pellet was discarded. The filtrate was centrifuged at 38,000 g for 30 min in the Sorvall RC 5B Plus (SS 34 rotor) at 4°C. The resulting supernatant was discarded, and the final pellet was resuspended in ~2 vol of ice-cold basic Hanks’ medium (pH 7.63) and aliquotted into 1.5-ml conical plastic centrifuge tubes. Membranes were frozen in liquid N2 and stored at −80°C until assayed within 2 wk of preparation. The yield of membrane protein was approximately 10–15 and 8–10 mg/g tissue for RM and WM, respectively. A modification of the 5′-nucleotidase assay was used to assess the efficiency of the membrane isolation (40). Enzyme activity was 3.5- to 5-fold higher in the isolated muscle membrane preparation compared with the crude muscle homogenate.

β-AR characterization. Specific binding assays used established and validated methods previously employed for trout ARs (5). Frozen membranes were thawed on ice. Aliquots of the membrane samples were assayed for protein using the bicinchoninic acid (BCA) assay (Sigma, St. Louis, MO) with BSA as standard and a SPECTRAMax PLUS 384 (Molecular Device, Sunnyvale, CA) microplate spectrophotometer. Protein concentrations were adjusted to 250–350 μg/mL based on preliminary experiments showing this concentration provided optimal binding. The radiolabeled, hydrophilic, mixed β-AR antagonist (−43-(t-butylamino)-2-hydroxypropoxy)-[5,7-3H]benzimidazol-2-one (1H)-CGP-12177A, referred to as [3H]CGP; Amersham Canada, Oakville, ON, Canada; specific activity 46.0 Ci/mmol) was used to characterize β-AR binding sites. Fifty microliters of trout RM and WM membranes (250–350 μg protein) were incubated in 5-ml polystyrene round-bottom clear tubes (Falcon) for 60 min, a time found to give optimal specific binding, at room temperature (~19°C) in a final volume of 150 μl and in the presence of varying concentrations of [3H]CGP (approximately 0.1 to 5 nM) to estimate total binding, while nonspecific binding was determined in the presence of 10 μM CGP-12177A (CGP, Sigma). All incubations were performed in basic Hanks’ (pH 7.63). Binding assays were terminated by aspirating the incubations through a cell harvester (Brandel 24R) onto prerinised (ice-cold 0.9% NaCl) borosilicate filters (no. 32 Mandel Scientific) and repeated washing (3×) with ice-cold 0.9% NaCl. The membranes collected onto the borosilicate filters were then placed in polyethylene scintillation vials containing 4 ml scintillant cocktail (Safety-Solve; Research Products International, Mount Prospect, IL). The vials were left in the dark for at least 24 h, and the radioactivity was determined using a Beckman Coulter LS650-multiscintillation counter using automatic quench correction.

Competition assays using 50 μl of muscle membranes (containing 200–300 μg protein) were incubated as above, in the presence of a constant concentration of 1 nM [3H]CGP (~14,500 dpm). Displacement of [3H]CGP was determined in the presence of five concentrations (10, 1, 0.1, 0.01, and 0.001 μM) of β-adrenergic antagonists [(±)-ICI-118,551 (ICI), atenolol (ATL), (±)-CGP, propranolol (Prop)] or β-AAs [(−)-dobutamine (Dob), propranolol (Proc), clenbuterol (Clen), ractopamine (Ract), BRL-37,334 (BRL), CL-316,243 (CL), (−)-Epi, and (−)-NE (all from Sigma) except for Ract, provided by Eli-Lilly (Greenfield, IN) and CL, provided by Dr. J. Himms-Hagen, Dept. of Biochemistry, Microbiology, and Immunology, Faculty of Medicine, Univ. of Ottawa] and terminated after a period of 60 min at room temperature. All subsequent manipulations were done exactly as stated in the specific binding assay methodology with the exception that all agonists were kept in tubes wrapped with aluminum foil and assays were carried out in subdued light.

β-AR coupling to ACase. ACase activity was assayed by adapting methods described for eel liver membranes (10, 12), rockfish enterocyte and brain membranes (26), and rat liver membranes (43). Membrane protein (45–55 μg) was incubated in medium (in mM: 12.5 MgCl2, 5.0 ATP, 20 creatine phosphate, 0.025 GTP, 6.0 theophylline, 50 Tris-HCl, and 6 U/ml creatine phosphokinase and 0.1 mg/ml BSA) in the presence of various agonists (l-isoproterenol (iso), Clo, Ract) and antagonists [Prop, (±)-CGP] and the ACase activator forskolin (FSK). The agonist Prop was preincubated for 10 min to ensure proper blocking of the ARs. After 10 min at room temperature (~19°C), the reaction was stopped by immersing the tubes in boiling water for 3 min. The samples...
were subsequently frozen in liquid N₂ and stored at −80°C until assayed for cAMP content within 1 wk. As above, the agonists were kept in tubes wrapped with aluminum foil, and assays were carried out in subdued light to prevent photo-degradation.

Proteins were precipitated by centrifugation (14,000 g, 5 min, Beckman-Coulter Microfuge R, F241.5 rotor at 4°C). cAMP was determined in supernatants after a modified method that extended the enzyme immunoassay kits from Amersham (Mississauga, ON, Canada) (T. P. Mommsen, personal communication). This included diluting the antiseraum (2×) with additional 3,3',5,5'-tetramethylbenzidine substrate solution (TMB substrate; Sigma) and purchasing an additional anti-rabbit IgG-coated plate (Cayman Chemical, Ann Arbor, MI). This permitted doubling the number of analyses per cAMP kit, and preliminary studies validated this procedure. The plates were read at 450 nm with a SPECTRMax PLUS 384, and the cAMP production was expressed as picomoles cAMP per milligram protein per 10 minutes. Membrane protein was assayed as previously described using the BCA protein assay.

Statistics. Receptor saturation and competition data were analyzed using the EBDA and LIGAND computer programs (28). All further data conversions used Microsoft Excel 2000, graphs were plotted using SigmaPlot 2000 (SPSS, Chicago, IL), and statistical differences were evaluated using appropriate tests with SigmaStat 2.0 (SPSS). A value of P < 0.05 was accepted to indicate significant differences.

RESULTS

β-AR characterization. The affinity (Kd) and number (Bmax) of β-adrenergic binding sites on rainbow trout RM and WM membranes were determined by incubating isolated membranes with increasing concentrations of [3H]CGP (Fig. 1, A and B, respectively). In both tissues, specific binding increased as the concentration of radiolabeled ligand increased to eventually saturate at ~1.5 nM. Specific binding was higher than nonspecific binding up to concentrations near 4 nM and was approximately 0.5 to 3% of total radiolabeled ligand (maximum around 0.5–1 nM of radiolabeled ligand). Nonspecific binding increased linearly and was between 30 and 60% of total binding (lower around 0.5 to 1 nM and higher around 5 nM). Scatchard analyses (Fig. 1C) were linear (EBDA; Ref. 28) and indicated the presence of a homogeneous class of binding sites in both tissues (LIGAND at P < 0.05; Ref. 28). Kd and Bmax values are presented in Table 1. A paired t-test revealed no significant difference between Kd values (P > 0.05) but a significant difference between Bmax values.

Competition assays were performed using classic mammalian AR agonists [Fig. 2, A (RM) and C (WM)] and antagonists [Fig. 2, B (RM) and D (WM)]. The mammalian antagonists classified as mixed β-agonists, CGP and Prop, displaced [3H]CGP to ~30% of total binding at the highest concentration used (10 μM). The β2-agonist ICI and the β2-agonists Clen and Ract were less effective and displaced to <50% of total binding. The endogenous adrenergic agonists Epi and NE both displaced to ~60% of total binding with a slightly higher displacement by Epi. The remaining agonists and antagonists displaced to <60% of total binding, although quantitative differences were noted between Dob (a β1-agonist) and Proc (a β2-agonist) displacement in RM and WM (Fig. 2, A and C).

The concentration of ligands causing 50% displacement of specific binding (Kd) was determined using
and in the presence of 1/100 M concentrations of production was assessed. Assays performed on trout RM and WM membranes, and cAMP [3H]CGP, [3H]CGP-12177A. *Significant difference between red and white muscle Bmax values (paired t-test, P < 0.05), but not Kd values.

EBDA (Table 2). The Kd values for the agonists were in the following order: Clen ≈ Ract > Proc ≈ NE ≈ Dob < Epi with no significant displacement by BRL or CL in the RM, and Clen ≈ Ract > Epi ≈ NE > Proc > BRL with no significant displacement by Dob or CL in the WM. The Kd values for the antagonists were in the following order: CGP > Prop > ICI, with no significant displacement by ATL in RM or WM membranes.

β-AR coupling to ACase. ACase assays were performed on trout RM and WM membranes, and cAMP production was assessed. Assays used increasing concentrations of β2-agonists [Fig. 3, A (RM) and B (WM)], and in the presence of 1 μM β2-agonists with and without 100 μM of the general β-antagonist Prop [Fig. 4, A (RM) and B (WM)]. Basal activities of RM cAMP production (means ± SE in pmol cAMP·mg protein−1·10 min−1) were 24.7 ± 3.1, and the β2-agonists significantly increased cAMP production at a concentration of 1 μM Clen and Ract (Fig. 3A). Similarly, WM basal values of cAMP production were 9.6 ± 3.5, and production increased significantly at concentrations of 0.1 and 0.01 μM Clen and Ract, respectively (Fig. 3B). The production of cAMP increased in a dose-dependent manner up to 10 μM Clen and Ract. The fold change in RM and WM membrane ACase activities was approximately 2.4 and 6.5, respectively, comparing basal with agonists at 10 μM agonist. This difference in fold increase is due to a significantly lower basal activity of ACase in WM compared with RM, as activities in the muscle membranes at 10 μM agonists were the same.

Incubating membranes with 1 μM β2-agonists (Clen, Ract, and Iso) significantly increased cAMP production in both RM (Fig. 4A) and WM (Fig. 4B) membranes. Preincubating with 100 μM Prop for 10 min completely blocked these significant agonist-induced increases. No significant differences existed between basal and Prop alone, or between basal, Prop alone, and Prop plus the β2-AAs (Clen, Ract, and Iso). Interestingly, there were no significant differences between any of the β2-AAs within a muscle type or between RM and WM; there

Table 1. Binding affinities and maximum number of binding sites for [3H]CGP (β-adrenoceptors) on rainbow trout red and white muscle membranes

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Kd (nM)</th>
<th>Bmax (fmol/mg protein)</th>
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<tbody>
<tr>
<td>Red muscle</td>
<td>0.14 ± 0.03</td>
<td>3.22 ± 0.11</td>
</tr>
<tr>
<td>White muscle</td>
<td>0.18 ± 0.03</td>
<td>2.60 ± 0.13*</td>
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All values are means ± SE; n = 6. Binding affinity (Kd) and maximum no. of binding sites (Bmax) determined from Fig. 1C. [3H]CGP, [3H]CGP-12177A. *Significant difference between red and white muscle Bmax values (paired t-test, P < 0.05), but not Kd values.

Fig. 2. Displacement curves representing competition between CGP and various adrenoceptor (AR) agonists and antagonists performed on rainbow trout RM [agonists (A) and antagonists (B)] and WM [agonists (C) and antagonists (D)] membranes. Assays contained between 250 and 350 μg protein/50 μl, and incubations were for 60 min at a constant concentration of 1 nM [3H]CGP in the absence (TB) and presence of 5 concentrations (10, 1, 0.1, 0.01, and 0.001 μM) of agonists (–)epinephrine (Epi), (–)-norepinephrine (NE), clenbuterol (Clen), ractopamine (Ract), procaterol (Proc), debutamine (Dob), BRL-37,334 (BRL), and CL-316,243 (CL) and antagonists (±)-ICI-118,551 (ICI), atenolol (ATL), propranolol (Prop), and CGP. Values represent means of 4 experiments (each experiment from an individual animal) done in duplicate. Variations (approximately ±5%) for individual data points are omitted for clarity.
membranes using various agonists and antagonists for [3H]CGP in rainbow trout red and white muscle illustrate the presence of β-adrenergic binding sites and coupling to cAMP in skeletal muscles. However, this study is the first characterization of β-adrenergic binding sites and coupling to cAMP in skeletal muscles of a teleost fish.

**DISCUSSION**

The main objectives of this study were to demonstrate the presence of β-ARs in RM and WM of the rainbow trout, pharmacologically determine the β-AR subtype(s) present, and show a causative association between β-AA binding and the activation of the subsequent signaling pathway. β-Adrenergic binding sites and increased cAMP production on β2-AA binding have been characterized in mammalian red (oxidative type I) and white (glycolytic type II) skeletal muscles. However, this study is the first characterization of β-adrenergic binding sites and coupling to cAMP in skeletal muscles of a teleost fish.

### β-AR characterization

The use of different radioactive ligands ([3H]CGP, [3H]dihydroalprenolol, and [125I]iodocyanopindolol) and different muscle preparations makes it difficult to compare these fish studies with previous studies. However, Jenson et al. (17) used membranes isolated from adult and juvenile rats and the radioligand [3H]CGP and reported similar results as shown here. In adult rat membranes, CGP affinity (K_d in nM) was 0.37 and 0.31 and the number of binding sites (B_max in fmol/mg protein) was 9.38 and 4.74 in red (soleus) and white (extensor digitorum longus) skeletal muscles, respectively. Similarly, these values in juvenile rat membranes were K_d values (in nM) of 0.27 and 0.24 and B_max values (in fmol/mg protein) of 11.21 and 5.45 in red (soleus) and white (epitrochlearis) skeletal muscles, respectively. Red muscle was, however, a significant difference between basal ACase activities in RM and WM as noted in Fig. 3, but this difference is eliminated by incubation of the membranes with Prop alone. The β-antagonist CGP was also tested with and without Prop, and no significant differences in cAMP production were observed in either membrane type (data not shown). Additionally, an ACase activator, FSK (10 μM), significantly increased cAMP production by approximately 7- and 14-fold above basal in RM and WM membranes, respectively (Fig. 4, A and B). This represents approximately a three-fold greater activation than observed for any of the agonists used. cAMP production in the presence of FSK was not significantly different between RM and WM membranes.

### Table 2. Competitive displacement parameters (K_i) for [3H]CGP in rainbow trout red and white muscle membranes using various agonists and antagonists

<table>
<thead>
<tr>
<th></th>
<th>Red muscle</th>
<th>White muscle</th>
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<tr>
<td>Agonists</td>
<td></td>
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<tr>
<td>Epi (β2-AR &gt; β1-AR)</td>
<td>3.298 ± 2.295</td>
<td>201 ± 61</td>
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<tr>
<td>NE (β1-AR &gt; β2-AR)</td>
<td>1.579 ± 790</td>
<td>332 ± 144</td>
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<tr>
<td>Clen (β2-AR)</td>
<td>332 ± 100</td>
<td>83.4 ± 10.0</td>
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<tr>
<td>Ract (β2-AR &gt; β1-AR)</td>
<td>110 ± 50.3</td>
<td>136 ± 103</td>
</tr>
<tr>
<td>Proc (β2-AR)</td>
<td>1,246 ± 363</td>
<td>710 ± 69</td>
</tr>
<tr>
<td>Dob (β1-AR)</td>
<td>1,902 ± 580</td>
<td>—</td>
</tr>
<tr>
<td>BRL (β2-AR)</td>
<td>—</td>
<td>1,185 ± 482</td>
</tr>
<tr>
<td>CL (β1-AR)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Antagonists</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CGP (nonselective β-AR)</td>
<td>3.0 ± 0.8</td>
<td>1.3 ± 0.8</td>
</tr>
<tr>
<td>Prop (nonselective β-AR)</td>
<td>37.3 ± 6.6</td>
<td>13.9 ± 1.7</td>
</tr>
<tr>
<td>ICI (β2-AR)</td>
<td>472 ± 54</td>
<td>203 ± 24</td>
</tr>
<tr>
<td>ATL (β1-AR)</td>
<td>—</td>
<td>—</td>
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</table>

All values are means ± SE; n = 4. Current pharmacological classification established for mammals is presented in parenthesis. Concentration of agonist or antagonist causing 50% displacement of specific binding (K_i) was calculated using the EBDA computer program (28). Agonists or antagonists causing <50% displacement of specific binding even at the highest concentration tested (10 μM) are represented by a dash. AR, adrenoceptor; Epi, epinephrine; NE, norepinephrine; Clen, clenbuterol; Ract, ractopamine; Proc, propranolol; Dob, dobutamine; BRL, BRL-37,334; CL, CL-316,243; Prop, propranolol; ICI, ICI-118,551; ATL, atenolol.
Fig. 4. Effects of 10 μM forskolin (FSK) and 1 μM concentrations of various adrenergic agonists [Clen, Ract, and (−)-isoproterenol (Iso)] with or without 10 μM concentration of the adrenergic antagonist Prop on RM (A) and WM (B) membrane adenylyl cyclase activities. Assays containing 45–55 μg protein were incubated for 10 min in the presence or absence of agonists, while those incubated with the antagonist Prop were preincubated for 5 min before 10-min incubation with the agonists. Means ± SE are presented for 4 independent experiments (each experiment from an individual animal) measured in duplicate. Levels of significance compared with basal or Prop alone and agonist plus Prop are represented by * and †, respectively (A and B). There is a significant difference in basal cAMP production between RM and WM membranes (paired t-test, P < 0.05).

skeletal muscle membranes had similar binding affinity but double the number of binding sites compared with WM in both adult and juvenile rats. Comparing these values reported in rats with those of trout RM and WM (Table 1) indicates slightly higher affinities in trout but approximately two- to five-fold lower B_max values. The trend of higher binding site numbers in RM compared with WM was retained, as reported in other mammalian studies that used other radiolabeled ligands and/or preparations (13, 21, 33, 45). Possible contamination by vascular tissue is considered to be an insignificant contributing factor to the higher binding site numbers in RM compared with WM because <3% of the RM volume is vascular tissue (6). The smaller number of cell surface hormone receptors in fish compared with mammals is a consistent observation reported for other receptor types (30).

Furthermore, comparing the values obtained from trout RM and WM (Table 1) with the literature for rainbow trout liver, heart muscle, and red blood cell β-ARs reveals some differences. Compared with the liver, RM and WM had slightly higher affinity (or lower K_d values) (approximately 0.16 vs. 0.4 nM) but three-fold lower B_max values (approximately 3 vs. 9 fmol/mg protein; Ref. 9). RM and WM had very similar K_d values to heart muscle (14, 15) but B_max values about eight-fold lower (3 vs. 24 fmol/mg protein; Ref. 14). RM and WM had much higher affinity (lower K_d values) than red blood cell (~0.16 nM vs. 2.5–4 nM; Refs. 16, 35). No comparable B_max values are available as the binding assays were performed on whole cells, and therefore values are expressed as number of binding sites per cell.

Despite the much higher number of binding sites in liver and heart muscle, skeletal muscle comprises ~50% of the total body weight (14) compared with ~1% for liver (5) and 0.15% for heart. This difference between tissue masses renders the skeletal muscle a metabolically important target for CAs that needs further attention.

Previous studies reported mainly β_2-ARs and possibly β_1-ARs on adult and juvenile rat skeletal muscles (17, 21) with some reports of β_3-ARs on mammalian skeletal muscle membranes (36, 39, 41). Competition assays using classic mammalian β-AR agonists and antagonists and rainbow trout RM and WM membranes revealed the presence of an atypical β_2-AR pharmacology (Fig. 2, Table 2). The order of potency for the antagonists (CGP > Prop > ICI with no displacement by ATL) clearly showed typical β_2-AR characteristics in both trout muscle membranes. The mixed β-AR antagonists CGP and Prop displaced the most effectively, closely followed by the β_2-AR antagonist ICI, but no displacement with the β_1-AR antagonist ATL. However, the order of potency for the agonists in both trout RM and WM was ambiguous and would suggest an atypical behavior. In RM (Clen ∼ Ract > Proc ∼ NE ∼ Dob ∼ Epi with no displacement by BRL and CL), the β_2-As Clen, Ract, and Proc displaced the most effectively, supporting the agonist result. The slightly higher affinity of NE compared with Epi supports β_1-AR characteristics (25). Also, some displacement by the β_1-AA Dob indicated the presence of β_1-AR characteristics. Similarly, in trout WM (Clen ∼ Ract > Epi ∼ NE > Proc > BRL with no displacement by Dob and CL), the β_2-As Clen and Ract displaced best, which again supported the results of the agonist experiments. However, the β_2-AA Proc would be ex-
pected to displace better than the endogenous CAs, but does not. Epi had a slightly higher affinity than NE, again supporting the presence of the β3-AR subtype. Also, some displacement by the β3-AA BRL would support a β3-AR component. This study is not the first to report ambiguities when using mammalian pharmacological agents in nonmammalian organisms, and this may indicate nonmammalian pharmacological classification (10).

The order of potency in hepatic membranes of the rainbow trout (Ref. 32; S. G. Dugan, personal communication) for the antagonists (CGP > ICI with no displacement by ATL) and the agonists (Clen > Epi > Proc > Ract with no displacement by NE and Dob) both supported a strict β2-AR pharmacology in the liver. Some quantitative differences exist between agonist and antagonist displacement in trout liver and muscle, but in general trends were similar. The most significant differences were much lower Kᵢ values for Ract in muscle (~120 nM) than liver (7,090 nM; S. G. Dugan, personal communication) and lower values for ICI and Proc displacement in the muscles compared with the liver. Also, NE displaced in both RM and WM but not in liver, and Dob displaced in RM but not in WM or liver. This may be evidence for the presence of more than one β-AR (β₁, β₂, and/or β₃-ARs) with similar affinities, because the Scatchard analysis (Fig. 1C) revealed only a single class of binding sites over the range of concentration used in the specific binding assays. In cattle, competitive ligand binding studies supported the presence of β₁- and β₂-ARs, but saturation analysis indicated one binding site without distinguishing between them (38). Furthermore, studies in pig and rodent reported Ract to have slightly less affinity for β₁ compared with β₂-ARs (4, 27). The higher CGP affinities found in RM and WM compared with liver that appeared to be exclusively β₂-AR support the existence of more than one muscle β-AR subtype. In fact, molecular evidence indicates the expression of a rainbow trout putative β₂-AR in liver and in both RM and WM (32), while a rainbow trout putative β₃-AR is expressed in RM and WM but not liver (J. G. Nickerson, personal communication). Therefore, the results of the agonist displacement studies need to be evaluated with caution, as it is possible that more than one β-AR exists in fish muscle as in mammalian skeletal muscle. Also, the coupling of these different subtypes to the signal transduction pathway could be different as demonstrated in rat muscle (37).

Interestingly, the two β₂-As, Clen and Ract, used in studies to enhance muscle growth in the meat industry (mammals and fish; Refs. 2, 29, 43, 44) displaced the radiolabeled ligand [³H]CGP with high affinity. Binding of these two β₂-As to rainbow trout muscle membrane β-ARs would imply possible direct effects of these growth supplements on skeletal muscle and the need for further investigations of these agents for use in the aquaculture industry.

β-AR coupling to ACase. To establish coupling between β-AR occupancy and the receptor transduction pathway, the production of cAMP was determined. This study used the ACase/cAMP assay on muscle membrane preparations (similar to studies done in hepatic membranes of fish; Refs. 8, 9, 11, 12) rather than whole muscle or transverse muscle slices commonly used in mammalian studies due to the anatomical differences between fish and mammalian skeletal muscles (18). As a result, direct comparisons of cAMP production rates between this study and the mammalian literature are difficult to make, but obvious qualitative comparisons are possible. Roberts and Summers (37), using soleus muscle slices from young rats, reported dose-dependent cAMP production with the β₂-AA (--) Iso with 50% maximum response (at 10 μM) reached at concentrations of 10–100 nM. Clen and Ract in both RM and WM membranes of the rainbow trout (Fig. 3, A and B, respectively) increased cAMP concentrations, but saturation at 10 μM was not achieved. The nonselective β-adrenergic antagonist Prop blocked cAMP production in RM and WM of trout (Fig. 4, A and B, respectively), as reported in mammalian studies. FSK, which directly stimulated mammalian ACase independent of the hormone receptor (47), activated mammalian ACase by seven- to eightfold at 10 μM compared with values reached using 10 μM Iso. RM and WM membranes from the trout are stimulated by FSK at a concentration of 10 μM about three-fold above values reached using 1 μM Clen, Ract, or Iso and 7- to 14-fold above basal cAMP production. The difference in potency of FSK between trout and mammalian ARs may reflect the specificity of FSK for ACase in the two experiments but also the tissue preparation, optimization of the various agents in the two preparations, the coupling between the AR and ACase, and the actual amount of ACase.

Studies in fish liver reported basal ACase/cAMP activities (in pmol cAMP·mg protein⁻¹·10 min⁻¹) of 20, 40, and 7.6 and stimulated activities (1–10 μM Epi) of 40–50, 63, and 20.5 in American eel (10, 12), bullhead catfish (8), and rainbow trout (9), respectively. In comparison, basal values in trout RM were similar to eel while WM basal values were more similar to rainbow trout liver activities. Stimulated values (1 μM Iso) in RM and WM were similar to bullhead liver stimulated values. These levels of agonist-stimulated cAMP production in RM and WM were obtained with 10 times less agonist compared with liver. As the number of liver β₂-ARs is about three-fold higher per gram tissue than in the muscle, the coupling between the ARs and ACase in muscle may be altered compared with that in liver.

It is interesting that RM had significantly higher basal rates of ACase activities compared with WM. However, this trend was abolished in samples incubated with the antagonist Prop and the ACase activator FSK. We know of no similar observation in the mammalian literature, but coupled with the higher number of β-ARs in RM, it may indicate a greater sensitivity or amplification of the message conveyed by Epi, NE, or any β-AA to ACase in RM than in WM.

In conclusion, rainbow trout RM and WM membranes possess a single class of saturable β-adrenergic
[3H]CGP binding sites. A significantly greater number of CGP binding sites are located in RM compared with WM. These binding sites had an atypical β2-AR pharmacology as determined using mammalian β-adrenergic agonists and antagonists. This result implicates the presence of more than a single β-AR subtype in these muscles as preliminary molecular biology evidence indicates. The rainbow trout muscle β-AR transduces its cellular message through a G protein, ultimately activating the ACaSe/cAMP pathway. The increased cAMP production on stimulation with β2-AAs was dose-dependent and blocked using the antagonist Prop.

Perspectives

This study is the first to report the presence of functional β2-ARs and the causative association between β2-AA binding and subsequent receptor specific transduction in RM and WM of a fish. The precise effects of reduced or elevated levels of the second messenger cAMP need further investigation especially with respect to changes in muscle protein synthesis and degradation. Recent studies, mostly in mammals and a few in birds, reported increased protein synthesis and decreased protein degradation, resulting in an increased muscle protein accretion after treatments with β2-AAs. In mammalian muscle, convincing evidence exists that the effects of β2-AAs are mediated directly on the target tissues through a β2-AR, and the intricate details of the cellular signaling pathway involved are being investigated, including cAMP, protein phosphorylation (via protein kinase A), and activation of CREs by CREB (3, 25, 27). The presence of a functional muscle β2-AR system in the rainbow trout provides an excellent fish model to evaluate the impact of β2-AA treatments on fish muscle growth. Finally, this study provides important basic knowledge of β-AR function in the muscles of an early branching vertebrate and suggests potentially beneficial applications to the aquaculture industry.

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