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Structure/function studies on the activation of the rainbow trout melanocortin-2 receptor

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ABSTRACT

Functional expression of the rainbow trout (rt) melanocortin-2 receptor (MC2R) in CHO cells requires co-expression with a teleost melanocortin-2 receptor accessory protein (MRAP) such as zebrafish (zf) MRAP. Transiently transfected rtMC2R/zfMRAP1 CHO cells were used to evaluate the efficacy of alanine substituted analogs of hACTH(1-24) in three motifs in the ligand: $H^{6}F^{7}R^{8}W^{9}$, $G^{10}K^{11}P^{12}V^{13}G^{14}$, and $K^{15}K^{16}R^{17}R^{18}P^{19}$. Alanine substitution at all positions in each motif either completely blocked activation of the receptor ($H^{6}F^{7}R^{8}W^{9}$ and $K^{15}K^{16}R^{17}R^{18}P^{19}$) or resulted in just over 400 fold increase in EC_{50} value ($G^{10}K^{11}P^{12}V^{13}G^{14}$). Single alanine substitutions in the $H^{6}F^{7}R^{8}W^{9}$ motif indicated that substitution at either W^{9} or R^{8} resulted in a much larger increase in EC_{50} values as compared to substitutions at either F^{7} or W^{9}. Alanine substitution at either $K^{15}K^{16}$ or $R^{17}R^{18}P^{19}$ in the $K^{15}K^{16}R^{17}R^{18}P^{19}$ motif resulted in a statistically equivalent increase in EC_{50} value of at least 600 fold. Finally, alanine substitutions in the $G^{10}K^{11}P^{12}V^{13}G^{14}$ motif resulted in increases in EC_{50} values presumably as a result of altering the secondary structure of the ligand. However, truncated analogs of hACTH(1-24) in which either $G^{10}G^{14}$ (ACTH(1-22), or $K^{11}P^{12}V^{13}$ (ACTH(1-21) were removed had no stimulatory activity. Finally, some of the hACTH(1-24) analogs were tested using rainbow trout head kidney pieces *in vitro* to confirm whether the response to analogs seen with the transient transfected rtMC2R CHO cells was similar to that of trout interrenal cells. The results of these alanine substitution analog studies are used to construct a multistep hypothetical model for the activation of teleost and tetrapod MC2Rs to account for the unique ligand selectivity of this receptor.

1. Introduction

In teleosts the synthesis of the glucocorticoid cortisol is regulated by the activity of the hypothalamus/anterior pituitary/interrenal (HPI) axis (Wendelaar Bonga, 1997). In this axis, corticotropic cells in the anterior pituitary release the melanocortin, ACTH (adrenocortiocotropin) (Donaldson, 1981; Hontela, 2005) which in turn binds to the "ACTH" receptor on interrenal cells. The result is the synthesis and release of cortisol (Mommsen et al., 1999). In teleosts, as in mammals (Mountjoy et al., 1992), and most likely all other bony gnathostomes, the "ACTH" receptor is the melanocortin-2 receptor (MC2R). Orthologs of MC2R have been characterized from the genomes of six teleosts, including: *Takifugi rubripes* (Klovins et al., 2004), *Tetraodon nigroviridis* (Klovins et al., 2004), *Cyprinus carpio* (Metz et al., 2005), *Oncorhynchus mykiss* (Aluru and Vijayan, 2008), *Danio rerio* (Agulleiro et al., 2010), and *Sparus aurata* (Agulleiro et al., 2013). The unifying features of these studies are: (1) teleost MC2Rs cannot be functionally expressed in heterologous mammalian cell lines unless the accessory protein, MRAP1 (melanocortin-2 receptor accessory protein 1) is co-expressed (Agulleiro et al., 2010, 2013; Liang et al., 2011); and (2) either in *in vivo* or *in vitro* analyses, teleost MC2Rs can be activated by mammalian ACTH(1-24) but not by α-MSH (Metz et al., 2005; Aluru and Vijayan, 2008; Agulleiro et al., 2010). It should be noted that while functional expression of teleost and tetrapod MC2R orthologs requires co-expression with MRAP1, these MC2R orthologs cannot be effectively activated when co-expressed with MRAP2, a paralog of MRAP1 (Sebag and Hinkle, 2009; Webb and Clark, 2010).

Recent studies indicate that the interrenal cells associated with the head kidney in rainbow trout express MC2R (rtMC2R), the melanocortin-4 receptor (MC4R), and the melanocortin-5 receptor (MC5R) (Haitina et al., 2004; Aluru and Vijayan, 2008). However,

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Table 1
Comparison of rainbow trout and human ACTH(1-39).

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          [-------ACTH(1-24)-----]

rtACTH  SYSMEHFRWGKPVGRKRRPVKVYTNGVEEESSEAFPSEM

hACTH   SYSMEHFRWGKPVGKKRRPVKVYPNGAEDESAEAFPLEF
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Deduced rainbow trout(rt)ACTH(1-39)sequence (accession number: NP_001118190.1), and deduced human (h) ACTH(1-39) sequence (accession number:CAG46625.1). The HFRW motif is shaded in gray, and the R/KRRP motif is underlined. Positions that are different with the 1-24 sequence of the two ACTH sequences are shaded in black (white letters).

while acute stimulation of interrenal tissue with ACTH, results in an increase in cortisol production, stimulation with α-MSH has no effect on cortisol production (Aluru and Vijayan, 2008). Apparently rtMC2R responds to amino acid motifs in ACTH that are either absent from α-MSH or are oriented in a manner that prevents α-MSH from interacting with rtMC2R. Based on these observations, the goal of this study was to analyze the structure/function relationships between ACTH(1-24) and rtMC2R by expressing rtMC2R in Chinese Hamster Ovary (CHO) cells, and stimulating these transfected cells with various alanine-substituted analogs of hACTH(1-24). A recent study indicated that alanine substitutions of hACTH(1-24) in three amino acid motifs ($H^6F^7R^8W^9$; $G^{10}K^{11}P^{12}V^{13}G^{14}$; $K^{15}K^{16}R^{17}R^{18}P^{19}$) disrupted the activation of human MC2R expressed in (CHO) cells (Liang et al., 2013). The objectives of this study were to determine whether these alanine substituted analogs of hACTH(1-24) would produce the same effects on rainbow trout MC2R transiently expressed in CHO cells. A second objective of this study was to determine whether some of these analogs have the same effects on the stimulation of cortisol release by rainbow trout interrenal cells *in vitro*. As the primary sequences of human ACTH(1-24) and rainbow trout ACTH(1-24) are 92% identical (see Table 1), it seemed reasonable to use the human ACTH(1-24) analogs for this study.

2. Materials and methods

The rainbow trout (*O. mykiss*) MC2R (rtMC2R; accession number EU119870) was synthesized with a V-5 epitope tag, and the zebrafish (*D. rerio*) MRAP1 (zfMRAP1; accession number XP001342923.2) was synthesized with a FLAG epitope tag by GenScript (Piscataway, NJ). Both cDNA constructs were individually inserted in a pcDNA3.1+ vector for transfection into CHO cells.

The CHO cells were obtained from ATCC (Manassas, VA). Cells were grown in Kaighn's Modification of Ham's F12K media (ATCC) supplemented with 10% fetal bovine serum, 100 unit/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml normacin, and maintained in a humidified incubator with 95% air and 5% CO_2 at 37 °C. After the CHO cells reached 80% confluence, the cells were split into subcultures using 0.05% trypsin/0.53 mM EDTA.

Activation of rtMC2R was determined by using an indirect measure of cAMP production. For this assay, 2.5×10^6 CHO cells were co-transfected with rtMC2R, zfMRAP1, and CRE-Luc (luciferase reporter construct) (Chepurny and Holz, 2007) DNA constructs (2 μg each) using the Amaxa Cell Line Nucleofector II system (Lonza, MD) with solution T and program U-23 as described in Liang et al. (2011). The transfected CHO cells were seeded in a white 96-well plate (Corning Life Sciences, Manassas, VA) at a final density of 1×10^5 cells/well. Following a 48 h incubation at 37 °C, the transfected cells were stimulated with hACTH(1-24) analogs diluted in serum-free CHO media for 4 h. The hACTH(1-24) analogs were synthesized by New England Peptide (Gardiner, MA), and the list of analogs used in this study appears in Table 2. In brief, three sets of alanine-substituted analogs were analyzed: $H^6F^7R^8W^9$ analogs; $G^{10}K^{11}P^{12}V^{13}G^{14}$ analogs; and $K^{15}K^{16}R^{17}R^{18}P^{19}$ analogs. The hACTH(1-24) analogs were tested at doses ranging from 10^{-6} M to 10^{-12} M, and each dose was tested in triplicate and compared to a wild type hACTH(1-24) control. For this assay, the amount of cAMP produced per dose of ligand over a 4 h period is proportional to the expression of CRE/luciferase construct over that same time period. Hence, by adding luciferase substrate reagent (Bright GLO; Promega, WI) to each well as described in Liang et al. (2011), the amount of luminescence detected 5 min after adding the substrate will be a function of the level of the *de novo* luciferase in the stimulated cells. Luminescence was measured using a Bio-Tek Synergy HT plate reader (Winooski, VT). To determine the basal levels of cAMP production, transfected CHO cells stimulated with vehicle were measured along with each experiment group. Luminescence readings were corrected for basal cAMP levels and then the data for each dose response curve was fitted to the Michaelis–Menton equation to obtain EC_{50} values. Data were analyzed using Kaleidograph software (www.synergy.com). Data points are expressed as the mean ± standard error. All experiments were performed in triplicate. Differences between experimental treatments with corresponding controls were determined using unpaired two-tailed Student's *t*-test for equal variance. Significance was set at $P < 0.05$. The assays presented in this study were done prior to the cloning of rainbow trout MRAP [Sandhu, Dores,

Table 2
Human ACTH(1-24)alanine substitution analogs.

SYSME – $H^6F^7R^8W^9$ –	$G^{10}K^{11}P^{12}V^{13}G^{14}$ –	$K^{15}K^{16}R^{17}R^{18}P^{19}$ –VKVYP
$A^6F^7R^8W^9$	$A^{10}K^{11}P^{12}V^{13}A^{14}$	$A^{15}A^{16}R^{17}R^{18}P^{19}$
$H^6A^7R^8W^9$	$G^{10}A^{11}A^{12}V^{13}G^{14}$	$K^{15}K^{16}A^{17}A^{18}A^{19}$
$H^6F^7A^8W^9$	$G^{10}K^{11}A^{12}V^{13}G^{14}$	$A^{15}A^{16}A^{17}A^{18}A^{19}$
$H^6F^7R^8A^9$	$A^{10}A^{11}A^{12}A^{13}A^{14}$	
$A^6A^7A^8A^9$		

Alanine substitutions are highlighted in gray for the $H6F^7R^8W^9$ analogs, underlined for the $G^{10}K^{11}P^{12}V^{13}G^{14}$ analogs, or shaded in black for the $K^{15}K^{16}R^{17}R^{18}P^{19}$ analogs.