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Analysis of the pharmacological properties of chicken melanocortin-2 receptor (cMC2R) and chicken melanocortin-2 accessory protein 1 (cMRAP1)

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ABSTRACT

The chicken (*Gallus gallus*) melanocortin-2 receptor (cMC2R) can be functionally expressed in CHO cells when chicken melanocortin-2 receptor accessory protein 1 (cMRAP1) is co-expressed. The transiently transfected CHO cells responded in a robust manner to stimulation by hACTH(1-24) (EC_{50} value = 2.7×10^{-12} M +/− 1.3×10^{-12}), but the transfected CHO cells could not be stimulated by NDP-MSH at concentrations as high as 10^{-7} M. Incubation of cMC2R/cMRAP1 transfected cells with alanine substituted analogs of hACTH(1-24) at amino acid positions F^7 or W^9 completely blocked stimulation of the transfected cells. Similarly, incubation of cMC2R/cMRAP1 transfected cells with an analog of hACTH(1-24) with alanine substitutions at amino acid positions $R^{17}R^{18}P^{19}$ resulted in a 276 fold shift in EC_{50} value relative to the positive control ($p < 0.004$). Collectively these observations suggest that cMC2R has binding sites for the HFRW motif and KKRRP motif of hACTH(1-24), and both motifs are required for full activation of the receptor. While previous studies had shown that *Anolis carolinensis* MC2R and *Xenopus tropicalis* MC2R could be functionally expressed in CHO cells that co-expressed mouse MRAP1, co-expression of these non-mammalian tetrapod MC2Rs with cMRAP1 resulted in a significant increase in sensitivity to hACTH(1-24), as measured by EC_{50} value, for *A. carolinensis* MC2R ($p < 0.005$) and *X. tropicalis MC2R* ($p < 0.007$). The implications of these observations are discussed.

1. Introduction

The melanocortin receptors are a family of G protein-coupled receptors that are activated by the melanocortin-related polypeptides, ACTH, α-MSH, β-MSH, γ-MSH, and δ-MSH (Cone, 2006; Takahashi and Kawauchi, 2006). The later polypeptides are all derived from the polypeptide hormone/neuropeptide precursor, proopiomelanocortin (POMC) (Nakanishi et al., 1979), and as a result of differential posttranslational processing mechanisms these end-products are generated in a tissue specific manner in the pituitary and central nervous system of vertebrates (Vallarino et al., 2012).

The genomes of some teleosts and most likely all tetrapods encode five melanocortin receptor paralogous genes (i.e., mc1r, mc2r, mc3r, mc4r, and mc5r) (Klovins et al., 2004; Ling et al., 2004; Cone, 2006; Agulleiro et al., 2010). These receptors are involved in a number of physiological processes including regulation of pigmentation, glucocorticoid biosynthesis, appetite regulation, and exocrine gland secretion (Cone, 2006). From a pharmacological perspective the melanocortin receptors can be segregated into two groups based on ligand selectivity and functional expression in heterologous mammalian cell lines. Teleost and tetrapod MC1R, MC3R, MC4R, and MC5R can all be activated by either ACTH or the MSH-sized ligands. In addition, these melanocortin receptors can be functionally expressed in non-adrenal-derived cell lines such as HEK-293 cells or CHO cells (for review see Dores, 2013). By contrast, teleost and tetrapod MC2R orthologs (the ACTH receptor) can only be activated by ACTH, but not by any of the MSH-sized ligands including α-MSH (Mountjoy et al., 1992; Klovins et al., 2004; Ling et al., 2004; Agulleiro et al., 2010; Liang et al., 2011). In addition, MC2R can only be functionally expressed in HEK-293 cells or CHO cells if the accessory protein, MRAP1 (melanocortin-2 receptor accessory protein 1) is co-expressed (Metherell et al., 2005). MRAP1 is a single transmembrane integral protein that forms a homodimer. The MRAP homodimer forms a heterodimer complex with teleost and tetrapod MC2R orthologs to facilitate not only the trafficking of MC2R from the endoplasmic reticulum to the plasma membrane, but also to influence the 3-dimensional conformation of MC2R thus making

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it possible for ACTH to bind to and activate the MC2R/MRAP1 complex on the plasma membrane (Hinkle and Sebag, 2009; Webb and Clark, 2010).

Given the unique relationship between teleost and tetrapod MC2R and MRAP1, analyses of the pharmacological properties of MC2R orthologs were impeded prior to the discovery of MRAP1. For example, analysis of the *Gallus gallus* (chicken) genome project (Ling et al., 2004) indicated that five paralogous melanocortin receptor genes are present in the chicken genome. In this study, the MC1R, MC3R, MC4R, and MC5R paralogs could be individually functionally expressed in HEK293-EBNA cells, and ligand binding studies revealed that these receptors had a higher affinity for ACTH than for any of the MSH-sized ligands tested (Ling et al., 2004). In accordance with other studies from this period (Rached et al., 2005; Forti et al., 2006; Kilianova et al., 2006; Roy et al., 2007) chicken MC2R could not be functionally expressed in HEK293-EBNA cells. To circumvent this issue, Ling et al. (2004) expressed the MC2R paralog in M3 cells, a melanocyte cell line that endogenously expresses MC1R. Using a subtractive analysis protocol, Ling et al. (2004) observed that the MC2R transfected M3 cells had a more robust production of cAMP when stimulated with ACTH as compared to non-transfected M3 cells, and concluded that the higher cAMP levels following ACTH stimulation were the result of the functional expression of the MC2R paralog. Given the complexity of this assay (i.e., cMC1R can be activated by ACTH), the pharmacological properties of chicken MC2R were not further analyzed. Hence, while it is assumed that chicken MC2R can only be activated by ACTH, the definitive experiments could not be done.

With the subsequent discover of an MRAP1 ortholog in the chicken genome (accession # NM_001031515.1), we have been able to functionally express chicken MC2R in CHO cells by co-expressing chicken MRAP1 (cMRAP1) in these cells. This study provides a detailed analysis of the ligand selectivity properties of chicken MC2R as compared to other MC2R orthologs (i.e., human, reptile, and frog). In addition, the ability of chicken MRAP1 to facilitate the activation of these representative tetrapod MC2R orthologs co-expressed in CHO cells was also examined.

2. Material and methods

Chicken (*G. gallus*) MC2R (cMC2R; accession number NM_00103515.1) (Takeuchi et al., 1998) was synthesized with a V-5 epitope tag, and the chicken MRAP1 (cMRAP1; accession number NM_001031515.1) 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 Chinese Hamster Ovary (CHO) cells. The following mc2r cDNAs were also synthesized by GenScript with V5 epitope tags and individually inserted in a pcDNA3.1+ vector: human (hMC2R; accession number NM_000529.2), *Anolis carolinensis* (acMC2R; accession number XP_003215733), and *Xenopus tropicalis* (xtMC2R; accession number XP_002936118.1). Finally, mouse MRAP1 (mMRAP1; accession number NM_029844.3) was synthesized by GenScript with a Flag epitope tag and inserted in a pcDNA3.1+ vector.

CHO cells were obtained from ATCC (Manassas, VA) and 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. The cells were maintained in a humidified incubator with 95% air and 5% CO_2 at 37 °C. Once the CHO cells reached 80% confluence, the cells were split into subcultures using 0.05% trypsin/0.53 mM EDTA.

Functional activation of cMC2R was determined by using an indirect measure of cAMP production (CRE-Luciferase reporter assay (Chepurny and Holz, 2007)). In brief, 3.0×10^6 CHO cells were co-transfected with a cmc2r cDNA construct, a cmrap cDNA construct, and a CRE-Luciferase reporter construct. The three cDNA constructs (2 μg each) were transfected into CHO cells using the Amaxa Cell Line Nucleofector II system (Lonza, MD) in Solution T, utilizing 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) or the alanine-substituted analogs of hACTH(1-24) diluted in serum-free CHO media at concentrations ranging from 10^{-13} M to 10^{-7} M. Following a 4 h incubation, luciferase substrate reagent (Bright GLO; Promega, WI) was added to each well as described in Liang et al. (2011). The luminescence generated following a five minute incubation with the luciferase substrate 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 hACTH(1-24) and the alanine substituted analogs were synthesized by New England Peptide (Gardiner, MA), and the alanine substituted analogs used in this study are listed in Table 1.

3. Results

3.1. Comparison of MCR amino acid sequences

The alignment of the amino acid sequences of chicken melanocortin-2 receptor (cMC2R), selected tetrapod and teleost MC2R orthologs, and human melanocortin-4 receptor (hMC4R; Fig. 1) illustrates features common to melanocortin receptors, and also highlights the unique aspects of cMC2R relative to other MC2R orthologs. For all the MCR sequences presented in Fig. 1 there is at least 50% sequence identity in the following domains: IC1, TM2, IC2, TM6, EC3, and TM7. The high level of sequence identity in these domains is a characteristic feature of melanocortin receptors (Dores, 2009; Baron et al., 2009). In addition, the MC2R orthologs have six of the nine residues (marked by *) that are located in the HFRW binding site of human MC4R (Pogozheva et al., 2005).

Focusing just on the MC2R orthologs, there are two features that set cMC2R apart from the other tetrapod or teleost MC2R sequences presented in Fig. 1; the lengths of the N-terminal and C-terminal domains. All G protein-coupled receptors (GPCRs) have seven membrane spanning domains, three intracellular loops, and three extracellular loops, and an N-terminal domain and a C-terminal domain. However, the N-terminal domain of the melanocortin-2 receptors orthologs is the shortest in the GPCR superfamily (Horn et al., 2003). While the C-terminal of human MC4R has the YPX(3)L motif which may affect the sorting of this melanocortin receptor following internalization (Dores et al., 2012), it is possible that the extended C-terminal domain of cMC2R, although lacking the YPX(3)L motif, may also play a role in intracellular sorting.

Note that the N-terminal domain of cMC2R is considerably longer than the N-terminal domains of the tetrapod and teleost MC2R sequences presented in Fig. 1, and is closer in length to the N-terminal domain of human MC4R. Thus far the only MC2R ortholog with a comparably sized N-terminal domain is elephant shark MC2R (eMC2; Fig. 1). Studies on the functional expression of elephant shark MC2R transfected into CHO cells indicate that

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