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Inhibitory effects of β-endorphin on cortisol release from goldfish (*Carassius auratus*) head kidney: An *in vitro* study

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ABSTRACT

β-Endorphin (β-END) is an endogenous opioid peptide derived from the common precursor proopiomelanocortin, together with adrenocorticotropic hormone (ACTH) and melanocyte-stimulating hormone (MSH). Although the roles of ACTH and MSH in fish are well known, the roles of circulating β-END have not been elucidated. In the present study, we evaluated the biological roles of β-END in the goldfish. First, we cloned the cDNAs of the delta opioid receptor (DOR), kappa opioid receptor (KOR), and mu opioid receptor (MOR) from the brain of the goldfish. Second, we analyzed the tissues that expressed these genes by using reverse transcription polymerase chain reaction. Among the several tissues that contained the opioid gene transcripts, the mRNAs of DOR, KOR, and MOR were detected in interrenal cells of the head kidney, which produce cortisol. On the basis of these results, the effects of β-END on cortisol release were examined *in vitro*. β-END alone suppressed the basal release of cortisol in a dose-dependent manner. Moreover, β-END inhibited the cortisol-releasing activity of $ACTH_{1-24}$. Therefore, it is probable that the role of β-END in the interrenal cells is the suppression of cortisol release. Interestingly, the suppression of cortisol release was not observed with *N*-acetyl-β-END, indicating that acetylation decreases the activity of β-END in interrenal cells.

1. Introduction

Proopiomelanocortin (POMC) is a precursor protein for several peptide hormones, including adrenocorticotropic hormone (ACTH), melanocyte-stimulating hormone (MSH), and β-endorphin (β-END), which are generated in the pituitary gland (Takahashi and Kawauchi, 2006a,b). ACTH and MSH are collectively called melanocortins (MCs). These peptides are derived from POMC by cleavage of the processing signal, which comprises two basic amino acids. The pituitary is a major source of POMC, where the protein is synthesized in both the pars distalis (PD) and pars intermedia (PI); however, because of post-translational processing in a tissue-specific manner, a number of final products can be produced from POMC (Castro and Morrison, 1997; Eipper and Mains, 1980; Smith and Funder, 1988; Raffin-Sanson et al., 2003; Takahashi and Kawauchi, 2006a). The major products in the PD are ACTH and β-END, whereas end-products in the PI are α-MSH, which corresponds to *N*-acetyl $ACTH_{1-13}$ amide, and N-terminally acetylated and C-terminally truncated β-ENDs.

In mammals, the typical biological function of ACTH is the stimulation of the synthesis and release of corticosteroids from the adrenal cortex, and the function of α-MSH is melanogenesis (Eberle, 2000). In addition, MC peptides are associated with a variety of biological functions, as shown by the wide distribution of MC receptors in the brain and peripheral organs/tissues (Eberle, 2000). β-END is an endogenous opioid peptide that exhibits analgesic effects via opioid receptors (Li, 1981). Both the MC and opioid receptors are G protein-coupled receptors (GPCR), with seven transmembrane domains. Five subtypes of MC receptors – MC1R, MC2R, MC3R, MC4R, and MC5R – have been reported to date (Gantz and Fong, 2003; Mountjoy, 2000). For opioid receptors, four subtypes have been identified. Among these, the delta opioid receptor (DOR), kappa opioid receptor (KOR), and mu opioid receptor (MOR) mediate analgesia. The fourth member of the opioid receptor family, the nociceptin or orphanin FQ receptor (ORL), binds to the neuropeptide nociceptin or orphanin FQ but not to the opioid peptides (Dores et al., 2011; Stevens, 2011).

Moreover, in fish, the major biological roles of ACTH and MSH are regulation of corticosteroid synthesis and pigmentation, respectively, whereas MSH also exhibits weak but significant

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ACTH-like activities (Kobayashi et al., 2009, 2011a,b; Lamers et al., 1992; Rance and Baker, 1981). There are three types of α-MSHs, which differ in the number of acetyl groups added to the N-terminal Ser residues as follows: α-MSH has one acetyl group at the N-position of the Ser residue, and diacetyl (Di-Ac)-α-MSH has two acetyl groups at the N- and O-positions of the Ser residue; desacetyl (Des-Ac)-α-MSH does not contain an acetyl group (Eberle, 2000). The presence or absence of an acetyl group is associated with the roles of these α-MSH-related peptides on the chromatophores. The potencies with regard to their pigment-dispersing activities in flounder melanophores were as follows: Di-Ac-α-MSH > Des-Ac-α-MSH > α-MSH; however, these α-MSH-related peptides exhibited similar potencies in the xanthophores of flounder and goldfish (Kobayashi et al., 2011b, 2012b). Relationships similar to those observed between the number of acetyl groups and biological activities were also observed for ACTH-like activities (Rance and Baker, 1981; Lamers et al., 1992; Kobayashi et al., 2011a). These characteristics are thought to be linked to the differential presence of MC receptors in each cell (Kobayashi et al., 2010, 2011a, 2012a).

Compared to the growing body of knowledge on the biology of MC, we have only limited knowledge of the biological functions of β-END in fish; however, β-END has been shown to be associated with food intake and the immune system (de Pedro et al., 1995, 1996; Watanuki et al., 1999, 2000). Therefore, the present study was undertaken to provide insight into the biological role of β-END in fish, using goldfish as a model organism. For this purpose, we first cloned cDNAs for the DOR, KOR, and MOR. Second, we explored the tissues and organs expressing the genes for these receptors by reverse transcription polymerase chain reaction (RT-PCR). Finally, we examined the roles of β-END in cortisol release because transcripts of the three opioid receptors were detected in the total RNA prepared from the head kidney containing interrenal cells.

2. Materials and methods

2.1. Fish

Immature goldfish, *Carassius auratus*, were obtained from a commercial dealer in Shizuoka, Japan, and all experiments were conducted according to the Guidelines for the Care and Use of Animals of Kitasato University. The fish were reared in indoor tanks with circulating freshwater under a natural photoperiod. The average body sizes of the fish used for molecular cloning and gene expression tests were 4.9 cm in standard length (SL) and 3.9 g in body weight (BW). For these experiments, tissue samples were collected from fish anesthetized with 0.2% 2-phenoxyethanol and subsequently frozen in a dry ice/ethanol bath. Head kidneys used in the experiments for cortisol-releasing activities were collected from fish (14.3 cm SL, 93 g BW, on average) anesthetized with ice-cold water.

2.2. Peptides

Goldfish β-END$_{1-29}$ and *N*-Ac-β-END$_{1-29}$ were purchased from PHJapan (Hiroshima, Japan). ACTH$_{1-24}$ was synthesized and purified according to previously described methods (Takahashi et al., 1995). The amino acid sequence of ACTH$_{1-24}$, which is identical to those of barfin flounder ACTH-A, salmon ACTH-A, and tuna ACTH (Takahashi et al., 2005), had one residue that differed from the sequence of goldfish ACTH at position 20 (Ile in goldfish *vs.* Val in others) (Cerdá-Reverter et al., 2003).

2.3. Molecular cloning

2.3.1. Nucleic acid preparation for sequence determination

Total RNA was extracted from the brain using Isogen (Nippon Gene, Tokyo, Japan). First-strand cDNAs were synthesized from total brain RNA for amplification of DOR, KOR, and MOR cDNA with the SMART RACE cDNA Amplification Kit (BD Biosciences, Palo Alto, CA, USA). Custom oligonucleotides were synthesized at Nihon Gene Research Labs Inc. (Sendai, Japan).

2.3.2. Amplification of DNA fragments for sequence determination

PCR using a thermal cycler (MJ Mini; BIO-RAD, Hercules, CA, USA) under conventional conditions was performed to amplify the DNA fragments with the HotStar Taq Master Mix (Qiagen, Hilden, Germany) or TaKaRa LA Taq (Takara, Otsu, Japan). The amplification procedures were similar for all three receptor cDNAs. First, the middle segment of the cDNA of each opioid receptor was amplified from first-strand cDNA by PCR using HotStar Taq DNA polymerase, with primers designed based on the nucleotide sequences of the opioid receptors from other species. The 3′ region of cDNA was then amplified from first-strand cDNA by 3′ rapid amplification of the cDNA ends (3′ RACE) using HotStar Taq DNA polymerase, gene-specific primers, and the Universal Primer A Mix (UPM) provided in the SMART RACE cDNA Amplification Kit. The 5′ region of cDNA was amplified from first-strand cDNA by 5′ RACE using HotStar Taq DNA polymerase, UPM, and gene-specific primers. Finally, cDNA containing the full-length reading frame was amplified from first-strand cDNA by PCR using TaKaRa LA Taq DNA polymerase and gene-specific primers. The primer sequences are listed in Table 1.

2.3.3. Sequence determination and data processing

PCR-amplified DNA was purified by agarose gel electrophoresis (NuSieve GTG Agarose; Cambrex Bio Science, Rockland, ME, USA). DNA was extracted from the agarose gel using the QIAEX II Gel Extraction Kit (Qiagen), ligated into the plasmid pT7 Blue T-Vector (Novagen, Madison, WI, USA) or pSTBlue-1 AccepTor Vector (Novagen), and transfected into JM109-competent cells. Recombinant plasmid DNA was prepared using the alkaline-SDS method, and both strands were sequenced using a capillary DNA sequencer (3130-Avant Genetic Analyzer; Applied Biosystems, Foster City, CA, USA) via the BigDye Terminator Cycle Sequencing Ready Kit v3.1. DNASIS-Pro (Hitachi Software Engineering, Yokohama, Japan) was used to process nucleotide and amino acid sequences, calculate amino acid sequence identities, align amino acid sequences, and construct a phylogenetic tree by the neighbor-joining (NJ) method (Saitou and Nei, 1987). Transmembrane domains were predicted using a program for the prediction of transmembrane helices in proteins, "TMHMM Server v2.0" (http://www.cbs.dtu.dk/services/TMHMM-2.0/).

2.4. RT-PCR for tissue distribution

Various tissues (e.g., pituitary, brain, eyeball, gill, atria, ventricle, liver, spleen, intestine, body kidney, head kidney, skin, skeletal muscle, fat, and ovary) were obtained from three female fish. The testes were taken from three males. A sample of RNA was prepared using Isogen (Nippon Gene). RNA was then treated with TURBO DNase (Ambion, Austin, TX, USA) for 4 h at 37 °C. The RNA yield was estimated by spectrophotometry. For each tissue, an equal amount of total RNA (100 ng) from three individuals was combined and subjected to amplification using the One-Step RT-PCR Kit (Qiagen) with the primer sets shown in Table 2. β-Actin cDNA was used as a positive control. PCR products were electrophoresed