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Molecular characterization of calcitonin gene-related peptide (CGRP) related peptides (CGRP, amylin, adrenomedullin and adrenomedullin-2/intermedin) in goldfish (*Carassius auratus*): Cloning and distribution

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ABSTRACT

To further characterize the structure and function of calcitonin gene-related peptide (CGRP) related peptides in fish, we have cloned cDNA sequences for CGRP, amylin, adrenomedullin (AM) and adrenomedullin-2/intermedin (IMD) in goldfish (*Carassius auratus*) and examined their tissue distribution. CGRP, amylin, AM and IMD cDNAs were isolated by reverse transcription (RT) and rapid amplification of cDNA ends (RACE). The cloned sequences contain the complete four mature peptides, which present a high degree of identity with mature peptide sequences from other fish. Phylogenetic analyses show that goldfish AM and IMD form a subfamily within the CGRP-related peptides that is distinct from the CGRP/amylin sub-family. The distribution of goldfish CGRP-like peptides mRNA expression in different tissues and within the brain was studied by RT-PCR. CGRP, IMD and AM are detected throughout the brain, in pituitary and in most peripheral tissues examined. Amylin mRNA is mostly expressed in the brain, in particular posterior brain, optic tectum and hypothalamus, but is also present in pituitary, gonad, kidney and muscle. Our results suggest that goldfish CGRP, amylin, AM and IMD are conserved peptides that show the typical structure characteristics present in their mammalian counterparts. The widespread distributions of CGRP, AM and IMD suggest that these peptides could be involved in the regulation of many diverse physiological functions in fish. Amylin mRNA distribution suggests possible new roles for this peptide in teleosts, including the control of reproduction.

1. Introduction

The calcitonin/calcitonin gene-related peptide (CGRP) family consists of calcitonin, calcitonin gene-related peptide, adrenomedullin (AM), adrenomedullin-2 (or intermedin, IMD), amylin (or islet amyloid polypeptide, IAPP) and the recently identified calcitonin receptor-stimulating peptide (CRSP) [30,38]. Peptides of this family all have a six-amino acid ring structure (seven for calcitonin) close to their N-termini, formed by an intramolecular disulfide bond, which consists of the biologically active region of the peptides [32]. They all act through a calcitonin receptor-like receptor (CLR) complexed to

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a specific receptor-activity-modifying proteins (RAMPs) [5,13,27,28,31] and have diverse and sometimes overlapping physiological actions.

CGRP is a 37-amino acid neuropeptide derived from the tissue-specific alternative splicing of the calcitonin gene [51], which is processed to produce either calcitonin or CGRP. In mammals, CGRP is expressed in a number of tissues and exerts a wide range of biological effects including neuromodulation, vasodilatation and food intake and gastrointestinal regulation [23,33]. In fish, cDNAS encoding for CGRP have been isolated in a number of species including flounder (*Paralichthys olivaceus*) [39], medaka (*Oryzias latipes*) and pufferfish [30]. In fish, as in mammals, the calcitonin gene encodes both the calcitonin peptide and a CGRP peptide [4,30]. The calcitonin gene and CGRP-like molecules are highly expressed in the brain but also in other tissues such as the ultimobranchial and the pituitary glands [4,20].

Amylin is a 37-amino acid peptide hormone originally isolated from amyloid deposits from the pancreas of type II diabetic humans. In mammals, amylin is co-secreted with insulin from pancreatic beta cells in response to meals [53,54], binds to specific amylin receptors in the central nervous system (CNS) and exert various effects including suppressing glucagon secretion and slowing gastric emptying [54] and decreasing food intake and body weight [24,53]. In fish, IAPP has been identified in four species of teleosts, i.e. *Danio rerio* (zebrafish), *Salmo salar* (Atlantic salmon), *Myoxocephalus* (*cottus*) *scorpius* (daddy sculpin) [50] and pufferfish (*Fugu rubripes*) [3]. Amylin has been localized in fish endocrine pancreas, suggesting that it is produced in the periphery and perhaps transported to the brain where it has central actions [50].

AM is a 52-amino acid peptide initially isolated from porcine adrenal medulla [16]. The AM gene encodes for two bioactive peptides, AM and proAM N-terminal 20 peptide (PAMP) both generated by the post-translational enzymatic processing of preproAM [17]. In mammals, AM is produced by different cell types, in all tissues of the body, with the possible exception of the thyroid and thymus [14]. AM has a role in the regulation of nervous, endocrine, cardiovascular, renal and respiratory systems [22]. In mammals, AM is also involved in the control of feeding, acting as an anorexigenic signal [25,35,43]. To date, AM has been identified in two pufferfish species and zebrafish [29], rainbow trout [41], common carp (*Cyprinus carpio*) [19] and medaka [30]. In fish, AM mRNA is expressed in brain and several peripheral tissues [19,29] and AM immunoreactivity has been detected in the endocrine pancreas [21].

IMD/AM2 is a 47-amino acid peptide initially isolated in fish [29] and subsequently cloned by two independent groups in mammals [34,41]. In mammals, IMD is expressed in brain, pituitary and most peripheral tissues [40] and is involved in several physiological functions including cardiovascular and body fluid regulation [11,41], activation of the hypothalamic–pituitary–adrenal axis and central regulation of food and water intake [10,45]. In fish, IMD has been characterized in pufferfish and zebrafish [29], rainbow trout [41] and medaka [30]. In pufferfish, IMD is expressed in the brain and in the vascular-rich mesentery [29].

Little is known about the physiological roles of four peptides of the calcitonin/CGRP peptide family in fish. It has been suggested that CGRP might have a role in osmoregulation [20] and regulation of gut motility [49]. Amylin has a role in the regulation of feeding [47] and AM might be involved in cardiovascular regulation [1] in teleosts.

In order to further characterize the structure and putative function of calcitonin gene related-related peptides in fish, we have cloned cDNAs encoding for CGRP, amylin, adrenomedullin and intermedin complete mature peptides in goldfish and examined their tissue distribution in central and peripheral tissues.

2. Methods

2.1. Animals

Adult goldfish (*Carassius auratus*) of 12–27 g in weight were obtained from a commercial supplier (Ozark Fisheries, Stoutland, MO, USA) and were maintained (in St John's, NL, Canada) under a simulated photoperiod of 16L:8D in 60 l glass tanks, with constantly aerated and filtered water at 20 °C. Fish were fed a 1% wet body weight (bw) ration once a day with commercially prepared 2.5 mm × 1 cm cylindrical trout pellets (Corey Aquafeeds, Fredericton, NB, Canada). Fish were anesthetized before decapitation and dissection of different tissues. All experiments were carried out in accordance with the principles published in the Canadian Council on Animal Care's guide to the care and use of experimental animals.

2.2. Preparation of RNA

Goldfish were anesthetized in 0.05% tricaine methanesulfonate (MS-222; Syndel Laboratories, Vancouver, BC, Canada) and quickly killed by severing the spinal cord. Brain and peripheral tissues were dissected and placed in RNA later stabilizing solution (Qiagen, CA, USA) and stored at −20 °C. Total RNA from whole brain, pituitary, gill, heart, gut, liver, spleen, kidney, muscle, skin and gonads, and various brain areas, including olfactory bulbs, telencephalon and the preoptic region, hypothalamus, optic tectum-thalamus, cerebellum, posterior brain and spinal cord, were extracted from fresh tissues using TRIsure RNA isolation reagent (Bioline, London, UK) according to the manufacturer's protocol. Final RNA concentrations were determined by optical density reading at 260 nm.

2.3. Cloning of cDNAs by reverse transcription (RT) and rapid amplification of cDNA ends (RACE)

Primers used are listed in Table 1. To isolate CGRP, amylin and AM sequences, total RNA from brain was used. IMD cDNA was isolated from pituitary tissue. Extracted RNA was reverse transcribed at 42 °C for 1 h in a total volume of 20 μl consisting of 2 μg total RNA, 1 μl M-MLV RT buffer, 0.5 mM each dNTP, 0.5 μg oligo-dT-adaptor primer (dT-AP) and 200 U M-MLV Reverse Transcriptase (New England Biolabs, Pickering, ON, Canada).

In order to isolate cDNAs encoding CGRP and amylin homologs, primers were designed based on conserved regions in the sequences of CGRP and amylin in mammals and fish

Table 1 – Sequences of primers used for cDNA cloning and tissue distribution analysis

Primer	Sequence
CGRP	
Cloning primers	
CGRP1	5′-TACGACACAGAAGAGAGCCTGTA-3′
CGRP2	5′-CACAAATTTGCTGCTTCCAA-3′
Specific primers for 3′- and 5′-RACE	
3′-RC CGRP1	5′-GACTTCCTGAGCCGCTCAGG-3′
3′-RC CGRP2	5′-GCTCAGGGGGAATTGGAAGC-3′
5′-RC CGRP1	5′-CAGCAACCAAAGAAGAACTG-3′
5′-RC CGRP2	5′-CAAACGCCTGGGAGCCCACG-3′
5′-RC CGRP3	5′-GAGCCCACGTTTGTGGGGAC-3′
Specific primers for RT-PCR	
CGRPTD1	5′-CTTACTGGAATGGTCAGCAG-3′
CGRPTD2	5′-CGATCAGTGCTATCAACTGG-3′
Amylin	
Cloning primers	
Amyl 1	5′-GAAGTGCAACACAGCCACCT-3′
Amyl 2	5′-GTGGATCCTACGTTGGTTGG-3′
Specific primers for 3′- and 5′-RACE	
3′-RC-Amyl 1	5′-TGCGTGACTCAGAGATTAGC-3′
3′-RC-Amyl 2	5′-GACTTTCTCGTCCGCTCCAG-3′
5′-RC-Amyl 1	5′-TGTCTGCATTTGAACTATAG-3′
5′-RC-Amyl 2	5′-GGTGTTGGCTCCCACGTTGG-3′
5′-RC-Amyl 3	5′-GTTGGTCGGTGCGTAGACG-3′
Specific primers for RT-PCR	
AmyTD1	5′-ATACAAGCATTCCTGCCTGC-3′
AmyTD2	5′-CGACTGCAGCAAGTCTCTCT-3′
Adrenomedullin	
Cloning primers	
AM1	5′-GCCACATTCCAGCACTGACA-3′
AM2	5′-AGCAGCTCTTCCAGCTTGTG-3′
Specific primers for 5′-RACE	
5′-RC-AM1	5′-GTGACCCGTAGGGATTGATCT-3′
5′-RC-AM2	5′-GAGATCATGGAGACGGTGTGC-3′
5′-RC-AM3	5′-GTGTGCCAGCACATGCACCG-3′
Specific primers for RT-PCR	
AMTD1	5′-AGGGATTTGAGCCTTGCTGC-3′
AMTD2	5′-TCTCTGGAACGGACCTTCGC-3′
Intermedin	
Primers for 3′-based in other fish IMDs	
3′-RC-IMD1	5′-AGGTACAAAACCTCAGC-3′
3′-RC-IMD2	5′-TCAGCCATCGCCTCTACCAG-3′
Specific primers for 5′-RACE	
5′-RC-IMD1	5′-ACGCCTGTAAATTGTTAATTG-3′
5′-RC-IMD2	5′-CTTCTGGGATTGATTGGTGC-3′
5′-RC-IMD3	5′-GATTGGTGCGTCTTGCCGTC-3′
Specific primers for RT-PCR	
IMDTD1	5′-GGAACATTTCCGAGAACGTCGC-3′
IMDTD2	5′-TCTCGTGACAGGACGCCTGT-3′
Adaptor primers	
dT-AP	5′-GGCCACGCGTCGACTAGTAC(T17)-3′
AP	5′-GGCCACGCGTCGACTAGTAC-3′
Primers for internal control of RT-PCR	
EF1	5′-ATCACCAAGGAAGTCAGCGC-3′
EF2	5′-ACTCATGGTGCATCTCAACG-3′

(Table 1). PCR was carried out on first strand cDNA and PCR products were analyzed on a 2% agarose gel. The band of desired size was excised and purified and subcloned (see description below). Specific primers were then designed based on the cDNA fragments obtained above. For 3′-RACE, total RNA was reverse transcribed with dT-AP and the cDNA was amplified by two rounds of PCR, with dT-adaptor primer (dT-AP) and either 3′-RC-CGRP1 or 3′-RC-Amyl1, and adaptor primer AP and either 3′-RC-CGRP2 or 3′-RC-Amyl2 (Table 1), respectively. PCR products were then purified and cloned. For 5′-RACE, cDNA was synthesized by reverse transcription of brain total RNA with a specific primers (5′-RC-CGRP1 or 5′-RC-Amyl1) and tailed with poly(A) using terminal transferase (Invitrogen, Burlington, ON, Canada). Products were amplified by two rounds of PCR using dT-adapter primer and either 5′-RC CGRP2 or 5′-RC Amyl2 and AP and either 5′-RC-CGRP3 or 5′-RC-Amyl3, respectively. PCR products were then purified, cloned, and sequenced.

A small fragment of AM sequence was amplified using primers (AM1 and AM2) designed based on AM sequences from common carp, zebrafish and takifugu. For 5′-RACE, cDNA was synthesized by reverse transcription of brain total RNA with specific primers (5′-RC-ADM1) and tailed with poly(A) using terminal transferase (Invitrogen). Products were amplified by two rounds of PCR using dT-AP and 5′-RC-ADM2 and AP and 5′-RC-ADM3, respectively. PCR products were then purified, cloned, and sequenced.

To isolate the 3′-end of IMD cDNA, two primers, 3′-RC-IM1 and 3′-RC-IM2 were designed on the basis of regions of high identity among pufferfish, zebrafish, medaka and tetraodon sequences. For 5′-RACE IM cDNA was synthesized by reverse transcription of pituitary total RNA with a specific primers (5′-RC-IM1) and tailed with poly(A) using terminal transferase (Invitrogen). Products were amplified by two rounds of PCR using dT-AP and 5′-RC-IM2 and AP and 5′-RC-IM3, respectively. PCR products were then purified, cloned, and sequenced.

In all cases, the PCR reaction mixture was carried out in a total volume of 25 μl consisting of 1 μl PCR buffer, 0.2 mM each dNTP, 0.2 μM each primer, and 1 U of Taq polymerase (New England Biolabs). PCR products were separated by agarose gel electrophoresis, and the band of desired size was excised and purified using a GenElute™ gel purification kit (Sigma, Saint Louis, MI, USA). The desired PCR products were then subcloned using the pGEM-T vector system (Promega Corp., Madison, WI, USA). Plasmid DNA containing the DNA insert was purified by GenElute™ Plasmid Miniprep Kit (Sigma) and sequenced by the MOBIX Lab (McMaster University, Ontario, Canada).

2.4. Sequence analysis

DNA and deduced protein sequences were analyzed by the Basic Local Alignment Search Tool (BLAST) available from the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov). Multiple alignments of amino acid sequences were performed using ClustalW software (www.ebi.ac.uk/clustalw/). Phylogenetic tree was obtained by Neighbor Joining method with 1000 replications using Phylo Win software.

2.5. Detection of mRNA expression in goldfish brain regions and tissues

The distribution of CGRP, amylin, AM and IMD mRNA expression in different tissues and in discrete brain regions was studied by RT-PCR using goldfish elongation factor-1 α (EF-1α) as a control gene. Total RNA from brain, pituitary, gills, heart, gut, liver, spleen, kidney, muscle, skin and gonads and from distinct brain regions (olfactory bulbs, telencephalon, optic tectum-thalamus, hypothalamus, cerebellum, posterior brain and spinal cord) were isolated as described above. To ensure non-contamination with genomic DNA, 1 μg total RNA was reverse transcribed into cDNA using QuantiTect® Reverse Transcription kit (Qiagen, Crawley, UK) with integrated removal of genomic DNA contamination. PCR amplifications was carried out for 40 cycles with the pairs of specific primers CGRPTD1 and CGRPTD2, AmyTD1 and AmyTD2, AMTD1 and AMTD2, IMDTD1 and IMDTD2 (Table 1) for CGRP, amylin, AM and IMD, respectively. EF1 and EF2 were used as primers for the reference gene (Table 1). Primers were designed based on our sequences for each peptide and on goldfish elongation factor-1 α sequence (EF-1α, GenBank accession number AB056104). PCR products were run on agarose gels and visualized using the Epichemi Darkroom BioImaging System (UVP, Upland, CA, USA) equipped with a 12-bit cooled camera. Image processing and analysis were performed using LabWorks 4.0 software (UVP).

3. Results

3.1. Structure of the goldfish CGRP, amylin, AM and IMD

A 159-bp cDNA fragment of the goldfish CGRP gene (GenBank accession number EU000531) encoding a 38-aa CGRP putative mature peptide was cloned (Fig. 1A). The amino acid sequence of goldfish CGRP is identical to that of zebrafish CGRP and shares 78–100% identity with CGRPs from other fish species and 76–81% identity with human CGRP. Similar to CGRPs from other fish and mammals, goldfish CGRP presents a single disulfide bridge linking six amino acid residues and an amidation signal (glycine, G) at the C-terminus.

We have cloned a 500-bp cDNA sequence encoding for goldfish amylin (GenBank accession number EU000530), which includes a 380-bp open reading frame encoding for a putative proamylin of 126 aa that contains the complete putative mature peptide. Similar to other vertebrates, the mature goldfish amylin peptide is 38 aa (Fig. 1B). The goldfish peptide presents a 71, 78, 89 and 91% degree of identity with human, rat, salmon and pufferfish amylins, respectively. The mature goldfish amylin displays a six-amino acid ring structure close to the N-terminus formed by an intramolecular disulfide bond between two cysteine residues. A C-terminal composed of threonine–tyrosin–glycine (TYG), present in the other vertebrate amylins is also seen in goldfish.

Our 557 bp cloned AM cDNA (GenBank accession number EU000533) contains an open reading frame of 466 bp encoding a 50-aa complete putative mature protein (Fig. 1C). Goldfish AM shows identities of 65–95% and 44–46% with teleosts and mammals AM sequences, respectively. The mature goldfish

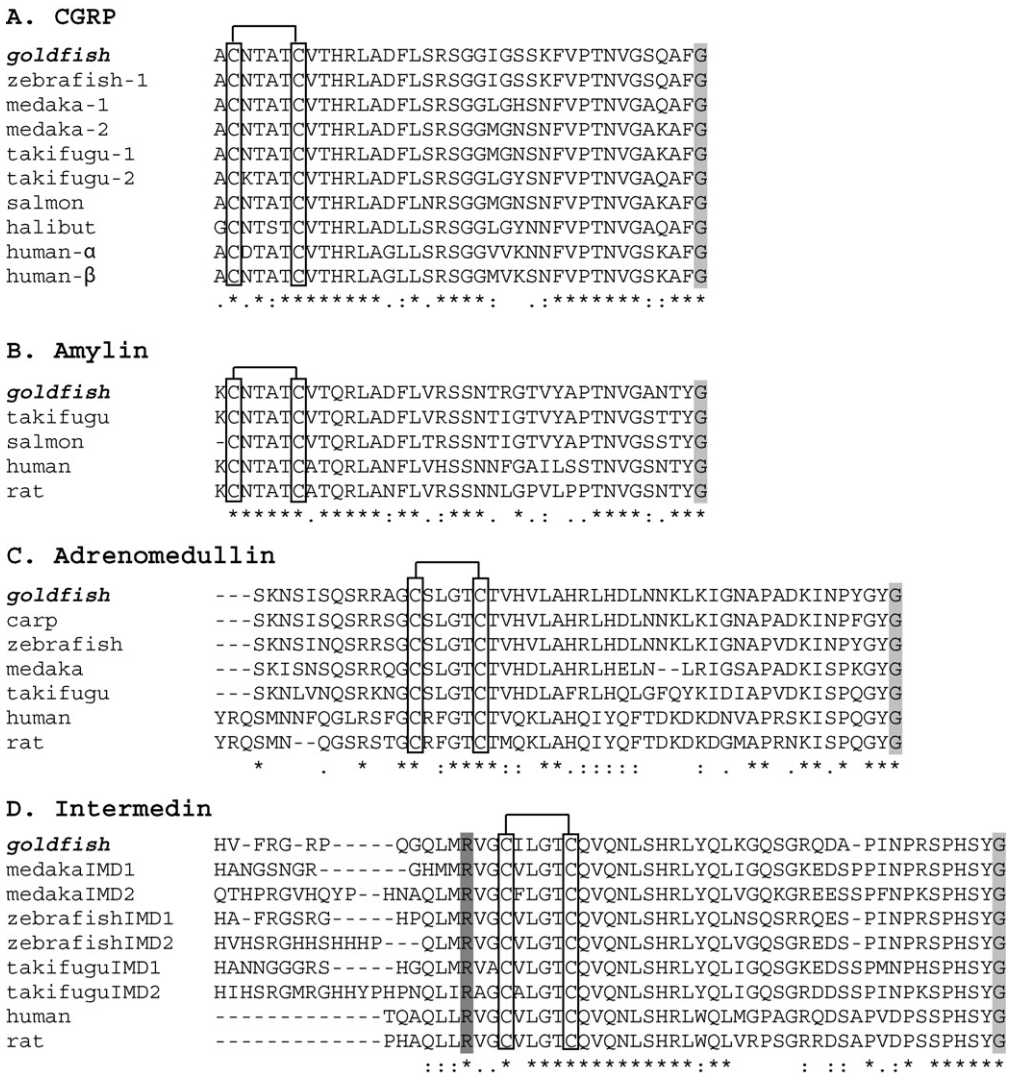

Fig. 1 – Sequence alignments for CGRP (A), amylin (B), adrenomedullin (C) and intermedin (D) putative mature peptides. Goldfish CGRP (GenBank accession no. EU000531) was compared to zebrafish (NP 001002471), medaka (GenBank accession no. 1-CGRP: AB257079; 2-CGRP: AB257080), takifugu (1-CGRP: Scaffold 2458; 2-CGRP: Scaffold 125), salmon (GenBank accession no. U71287), halibut (GenBank accession no. AB052782) and human (GenBank accession no. α-CGRP: X02330; β-CGRP: X02404). Goldfish amylin (GenBank accession no. EU000530) was compared to takifugu (Scaffold 662), salmon [50], human and rat (GenBank accession no. DQ516082 and NP 036718, respectively). Goldfish adrenomedullin (GenBank accession no. EU000533) was compared to carp (GenBank accession no. AB120940), zebrafish (GenBank accession no. XM693535), medaka (GenBank accession no. AB257074), takifugu (GenBank accession no. AB120295), human (GenBank accession no. D43639) and rat (GenBank accession no. D15069). Goldfish intermedin (GenBank accession no. EU000532) was compared to medaka (GenBank accession no. IM1: AB257075; IM2: AB257076), zebrafish (GenBank accession no. IM1: CK694927; IM2: AW421384), takifugu (GenBank accession no. IM1: AB120296; IM2: AB120297), human (GenBank accession no. AB121034) and rat (GenBank accession no. AB121036). The disulfide bond across cysteine residues is indicated by bracketed boxes. The amidation signal (G; Gly) are shadowed. The putative basic cleavage sites in IMD sequence are darkly shaded.

AM peptide presents the amidation signal (G) at the C-terminus and a six-amino acid ring structure close to the N-termini, which are both conserved among vertebrate AMs. The six-amino acid ring of teleost AMs displays two amino acid substitutions compared to mammalian AMs, arginine and phenylalanine (RF) residues being replaced by serine and leucine (SL), respectively, in the fish sequence (Fig. 1C). By examining the 5′ region of our cDNA fragment encoding goldfish preproAM and comparing it to that of zebrafish, carp, medaka, takifugu and human (Fig. 2), an enzymatic cleavage signal (KR), but no amidation site could be identified in the ''potential PAMP-like region'' of the goldfish sequence. This putative region is shorter than and presents only 21% identity with either human PAMP or takifugu PAMP-like sequences.

A cDNA fragment of 500 bp containing the complete putative mature IMD was also obtained (GenBank accession

Fig. 2 – Sequence alignments for goldfish (partial sequence), zebrafish, carp, medaka, pufferfish and human preproadrenomedullin. Mature proAM N-terminal 20 peptide (PAMP) in mammal and pufferfish PAMP-like peptide are shadowed. Boxes contain the predicted sequences for putative mature AM. Conserved amino acid sequences in the "possible PAMP-like region" in goldfish, zebrafish, carp and medaka are in bold letters. GenBank accession numbers are as in Fig. 1.

number EU000532). The amino acid sequence of the mature goldfish IMD is 53 aa long (Fig. 1D) and shares 77–90% identity with IMDs from other teleosts and 69–75% identity with mammalian IMDs. IMD proteins from fish are larger (53–61 aa) in relation to their mammalian counterparts (48 aa). Similar to other vertebrate IMDs, the goldfish peptide presents a single disulfide bridge with six amino acid residues formed by two cysteines as well as an amidation signal (G) at the C-terminus. Furthermore, goldfish IMD presents an arginine (R) residue seven amino acids downstream of N-terminus, which is conserved in all species.

The sequence alignments for goldfish CGRP, amylin, AM and IMD putative mature peptides reveal a low homology (Fig. 3A). However, the disulfide bond across cysteine residues and the amidation signal (Gly) at the C-terminus appear conserved in the four peptides. In goldfish, as in other vertebrates, the amino acid sequence of CGRP is close to that of amylin, the mature peptides sharing 57% identity. Goldfish AM and IMD share a 36% identity but only share 18–26% identity with goldfish CGRP and amylin mature proteins. Phylogenic analyses show that goldfish CGRP and amylin each groups with pufferfish and mammalian peptides, CGRP and amylin forming a sub-branch within this calcitonin peptide family. Similarly, goldfish AM and IMD each groups with fish and mammalian homolog peptides (Fig. 3B). Our data show that goldfish AM and IMD form a sub-family within the CGRP-related peptides that is distinct from the CGRP/amylin sub-family.

3.2. *Brain and tissue distribution of CGRP, amylin, AM and IMD mRNA*

RT-PCR was performed in order to localize mRNAs in different tissues and brain regions.

CGRP mRNA expression was detected in all tissues examined, i.e. brain, pituitary, gill, heart, gut, liver, spleen, kidney, muscle, skin and gonads (Fig. 4). Within the brain, CGRP has a wide range distribution and is expressed in all cerebral areas, with strongest signals in the posterior brain and spinal cord (Fig. 5).

Amylin mRNA is mostly expressed in the brain, but is also present in gonad (testis) and displays low expression levels in pituitary, kidney and muscle (Fig. 4). Within the brain, amylin is expressed in posterior brain, hypothalamus and optic tectum but not in olfactory bulbs, telencephalon, cerebellum, or spinal cord (Fig. 5).

AM mRNA expression was detected in brain, pituitary and all peripheral tissues examined, with the exception of gills (Fig. 4), and in all brain regions studied, with lowest levels in the spinal cord (Fig. 5).

IMD mRNA expression was detected in brain, pituitary and all peripheral tissues examined, with highest levels in brain, pituitary and gonads and lowest levels in gills, spleen and skin (Fig. 4). IMD is present in all brain regions tested (Fig. 5).

4. Discussion

4.1. *Structure of calcitonin gene-related peptide-related peptides in goldfish*

Goldfish CGRP shows a high percentage of identity with CGRPs from other fish and human CGRP. Mammals have two CGRP genes: one gene expresses both alpha CGRP and calcitonin, whereas the other expresses only beta CGRP [30]. Two CGRP genes are also present in fish, each producing one form of CGRP, CGRP-1 or CGRP-2 [30]. Although we only isolated one

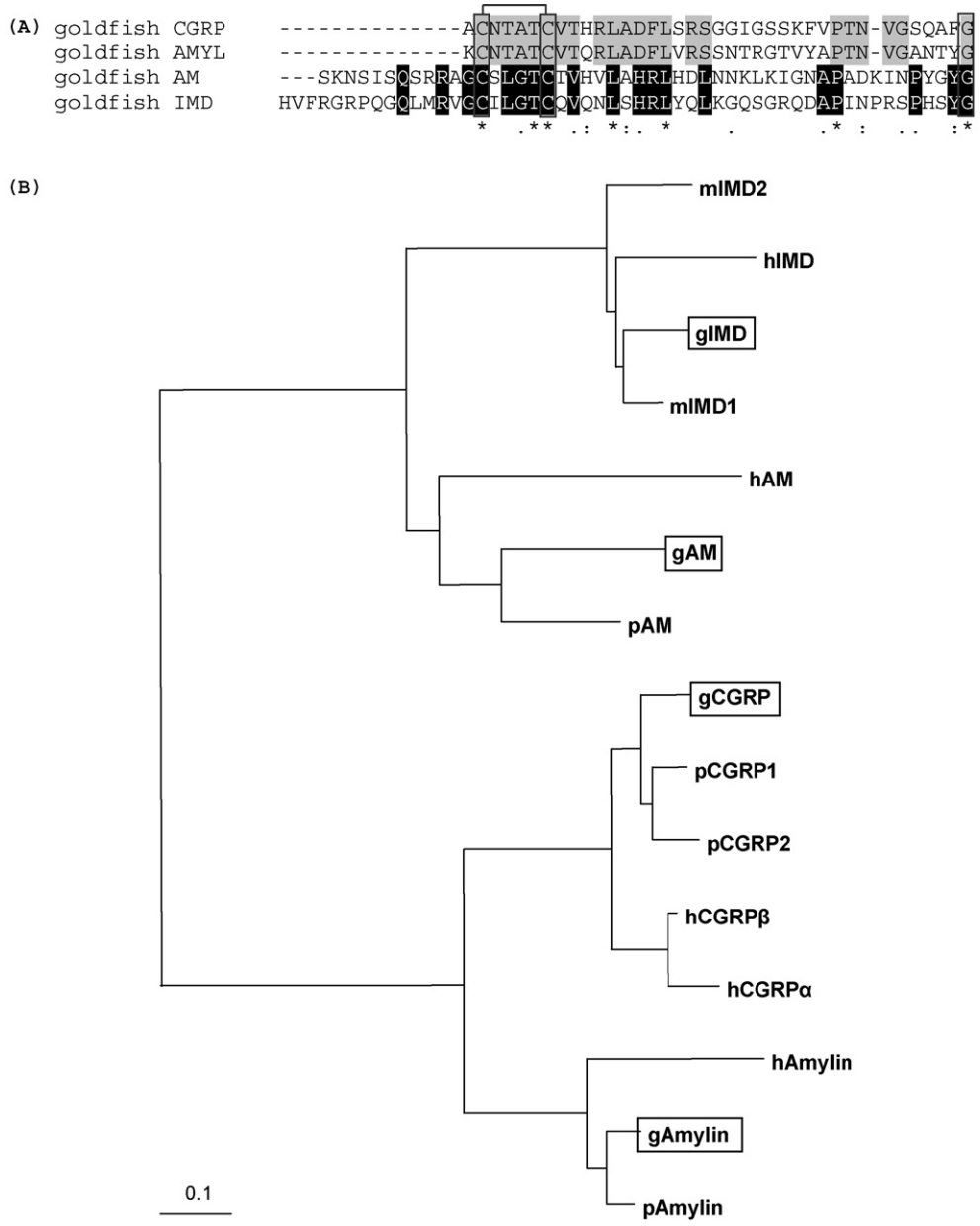

Fig. 3 – (A) Sequence alignments for goldfish CGRP, amylin (AMYL), AM and IMD putative mature peptides. The sequences conserved between CGRP and amylin are shadowed and sequences conserved between AM and IMD are blackened. The disulfide bond across cysteine residues is indicated by bracketed boxes. The amidation signals (G; gly) at the C-terminus are boxed. (B) Phylogenetic analysis of goldfish CGRP, amylin, AM and IMD putative mature peptides with CGRP-related peptides of other teleosts and human. The phylogenetic tree was obtained using the neighbor-joining method in Phylo Win. m, medaka; h, human; g, goldfish; p, pufferfish. The goldfish sequence is squared. GenBank accession numbers are as in Fig. 1.

form, it is probable that two CGRP forms exist in goldfish. Similar to other vertebrate CGRPs, goldfish CGRP is a 38-aa peptide. It contains two cysteine residues on positions 2 and 7, which form an intramolecular disulfide bond that is responsible for the 3D configuration of the biologically active peptide, and an amidation signal (Gly) at the C-terminus [5]. These same characteristics are also present in vertebrates amylins [6], including goldfish amylin.

To date, cDNAs encoding amylin have been identified in zebrafish (*D. rerio*), Atlantic salmon (*S. salar*), and pufferfish (*F. rubripes*) [3,50]. The mature goldfish amylin protein presents a high degree of identity with other fish amylins, as well as with mammalian amylins, suggesting that the mature peptide is highly conserved in vertebrates. We only found one form of goldfish amylin, which is consistent with the fact that only a single amylin ortholog appears to exist in fish, based on the *F. rubripes* genome [3].

As the previously described CGRP-related peptides, goldfish AM contain two cysteine residues that form a disulfide bond and an amidation signal (G) at the C-terminus [19,29,32,40,42].

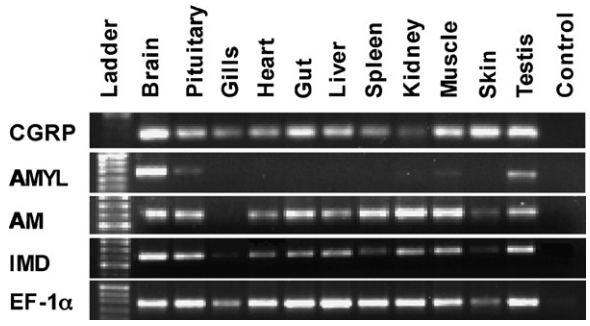

Fig. 4 – Distribution of CGRP, amylin (AMYL), adrenomedullin (AM) and intermedin (IMD) mRNA expression in whole brain, pituitary and peripheral tissues of goldfish as revealed by RT-PCR assays. EF-1α was used as reference gene. The RT-PCR products were of expected size with 148 bp for CGRP cDNA, 381 bp for AMYL cDNA, 295 bp for AM cDNA, 291 bp for IMD cDNA and 385 bp for EF-1α cDNA.

The mature goldfish AM protein presents a high degree of identity with other teleost AMs but only 46–48%, with mammalian AMs, suggesting that AM is highly conserved among fish but less among vertebrates. Moreover, arginine and phenylalanine (RF) residues present in the ring structure of mammalian AMs are substituted by serine and leucine (SL) residues in teleosts AMs, suggesting differences in receptor selectivity between different species. Five forms of AM, named AM1, 2, 3, 4 and 5, were originally reported in pufferfish [29] and later identified in zebrafish [29], rainbow trout [41] and medaka [30]. These five AMs seem to belong to three main subtypes, AM1/4, AM2/3, and AM5 [42]. Forms 1, 2 and 5 have also been isolated in mammals [42] suggesting that AM3 and AM4 could have been generated by gene duplications in fish. Mammalian AM2 actually corresponds to intermedin [34], so that this peptide is now referred to as AM2/IMD. Although we only isolated AM1 and AM2 (IMD), it is possible that other forms exist in goldfish.

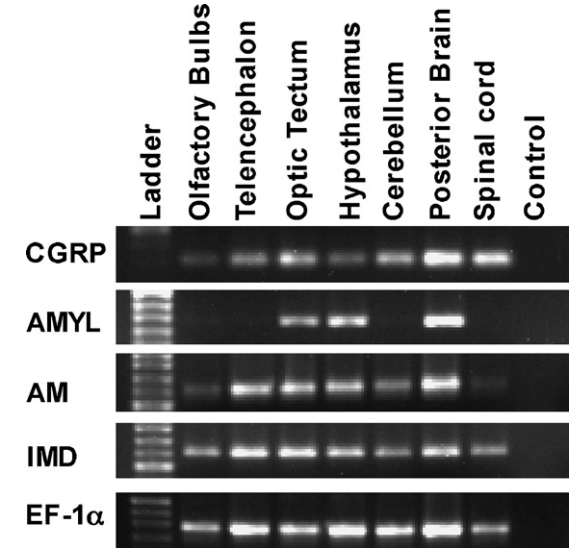

Fig. 5 – Distribution of CGRP, amylin (AMYL), adrenomedullin (AM) and intermedin (IMD) mRNA expression in discrete brain regions of goldfish as revealed by RT-PCR assays. EF-1α was used as reference gene. The RT-PCR products were of expected size with 148 bp for CGRP cDNA, 381 bp for AMYL cDNA, 295 bp for AM cDNA, 291 bp for IMD cDNA and 385 bp for EF-1α cDNA.

In mammals, proadrenomedullin contains proadrenomedullin N-terminal 20 peptide, a 20-residue sequence located upstream of the AM sequence. PAMP contains an amidation motif (RG) at the carboxy end and is followed by a proteolytic site (KR) [18,22]. PAMP and AM have similar physiological functions and both act as vasodilators, bronchodilators and regulators of hormonal secretion [37]. It has been suggested that a PAMP-like sequence exists just after the signal sequence of AM-1 in takifugu. This PAMP-like peptide displays a proteolytic site, but lacks the amidation motif at the carboxy end [29]. The putative PAMP-like region of the goldfish sequence displays a very low degree of identity with either human PAMP or takifugu PAMP-like sequence. As in carp [19], the goldfish sequence presents an enzymatic cleavage signal (KR) but no amidation site. Further studies will thus be required to determine if this sequence corresponds to a biologically active PAMP-like peptide.

Goldfish IMD presents a high degree of identity not only with other fish IMDs, but also with mammalian IMDs, suggesting that the mature peptide is highly conserved throughout phylogeny. In addition to the amidation donor residue (glycine, G) in the C-terminus, goldfish IMD also shows an arginine (R) residue close to the disulfide bond at the N-terminus, which is conserved among fish and mammals [3]. To date, IMD has been identified in five fish species (pufferfish, medaka, tetraodon, zebrafish and rainbow trout). Pufferfish, medaka and zebrafish have been shown to possess two IMD genes, IMD1 and 2 [29,30,41]. In mammals, two IMDs peptides, generated by proteolytic cleavage of the same preprohormone, have also been reported [34]. It is thus probable that a second IMD form exists in goldfish.

When comparing sequences of goldfish calcitonin-related peptides, the percentage of identity and phylogenetic analysis reveals that AM and IMD form a sub-family of peptides that is distinct from the CGRP/amylin sub-family, which is consistent with the previous studies by Ogoshi et al. on pufferfish [29].

4.2. *Tissue distribution of CGRP-related peptides in goldfish*

CGRP mRNA expression distribution in goldfish are in agreement with studies in mammals [48] and fish [2,7,9,12] showing that CGRP is expressed in many central and peripheral tissues and suggesting that this peptide could act as central neuropeptide and as neurotransmitter in the periphery. In mammals, amylin is synthesized from pancreatic beta cells, gastrointestinal tract, spinal cord and developing kidney [52,54]. In fish, amylin is present in the insulin-producing pancreatic cells (Brockmann bodies) of sculpin, and in salmon endocrine pancreas [50]. In goldfish, pancreatic tissue is scattered around the gut and the liver [8], but, interestingly, we found no amylin expression in either of these tissues. To our knowledge, this is the first report of the

presence of amylin in gonads of any vertebrate. The expression of amylin along the hypothalamic–pituitary–gonadal axis observed in goldfish might indicate a role of this peptide in the regulation of reproductive events. AM is present in the brain and most peripheral tissues of goldfish (this study), pufferfish [29] and common carp [19] as well as in pancreas of brown shark and rainbow trout [21]. The ubiquitous distribution of AM in fish, birds [55] and mammals [14] indicates that AM could act as a multifunctional regulatory peptide in vertebrates. The wide distribution of AM in goldfish brain is similar to that found in other vertebrate species [15,26,49] and suggests that AM also exerts central actions in fish as described in mammals [36,43,44]. The wide distribution of goldfish IMD contrasts with the IMD expression in pufferfish that is almost exclusively detected in the brain [29] and is consistent with the widespread distribution in mammals [34,41,45]. The high expression levels of IMD in goldfish brain and pituitary suggest that IMD might be involved in the central regulation of physiological functions and in the control of pituitary secretion in fish as reported in rodents [42,45,46].

In conclusion, cDNAs encoding four mature peptides from the calcitonin gene related family, CGRP, amylin, adrenomedullin and intermedin have been isolated in goldfish. Phylogenetic analyses show that these peptides are relatively well conserved among vertebrates but display species-specific differences in their tissue distribution. This study is a first step towards the characterization of these peptides whose physiological functions in fish remain for the most part unknown.

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REFERENCES

[1] Aota S. Cardiovascular effects of adrenomedullin in teleost fishes. Braz J Med Biol Res 1995;28:1223–6.
[2] Batten TF, Cambre ML. Calcitonin gene-related peptide-like immunoreactive fibres innervating the hypothalamic inferior lobes of teleost fishes. Neurosci Lett 1989;98:1–7.
[3] Chang CL, Roh J, Hsu SY. Intermedin a novel calcitonin family peptide that exists in teleosts as well as in mammals: a comparison with other calcitonin/intermedin family peptides in vertebrates. Peptides 2004;25: 1633–42.
[4] Clark MS, Bendell L, Power DM, Warner S, Elgar G, Ingleton PM. Calcitonin: characterisation and expression in a teleost fish, *Fugu rubripes*. J Mol Endocrinol 2002;28:111–23.
[5] Conner AC, Simms J, Barwell J, Wheatley M, Poyner DR. Ligand binding and activation of the CGRP receptor. Biochem Soc Trans 2007;35:729–32.
[6] Cooper GJ. Amylin compared with calcitonin gene-related peptide: structure, biology, and relevance to metabolic disease. Endocr Rev 1994;15:163–201.
[7] Domeneghini C, Radaelli G, Arrighi S, Mascarello F, Veggetti A. Neurotransmitters and putative neuromodulators in the gut of *Anguilla anguilla* (L.). Localizations in the enteric nervous and endocrine systems. Eur J Histochem 2000;44:295–306.
[8] Epple A. The endocrine pancreas. In: Hoar WS, Randall DJ, editors. Fish physiology, vol. II. New York: Academic Press; 1969. p. 275–319.
[9] Fouchereau-Peron M, Arlot-Bonnemains Y, Taboulet J, Milhaud G, Moukhtar MS. Distribution of calcitonin gene-related peptide and calcitonin-like immunoreactivity in trout. Regul Pept 1990;27:171–9.
[10] Fujisawa Y, Nagai Y, Miyatake A, Takei Y, Miura K, Shoukouji T, et al. Renal effects of a new member of adrenomedullin family, adrenomedullin2, in rats. Eur J Pharmacol 2004;1497:75–80.
[11] Fujisawa Y, Nagai Y, Miyatake A, Takei Y, Miura K, Shoukouji T, et al. Roles of adrenomedullin 2 in regulating the cardiovascular and sympathetic nervous systems in conscious rats. Am J Physiol Heart Circ Physiol 2006;290:H1120–7.
[12] Funakoshi K, Kadota T, Atobe Y, Nakano M, Tsukagoshi M, Goris RC, et al. Differential innervation of the goldfish tonic red muscles and twitch white muscles by neuropeptide-immunoreactive motoneurons. Brain Res Bull 2000;52: 547–52.
[13] Hay DL. What makes a CGRP2 receptor? Clin Exp Pharmacol Physiol 2007;34:963–71.
[14] Hinson JP, Kapas S, Smith DM. Adrenomedullin a multifunctional regulatory peptide. Endocr Rev 2000;21:138–67.
[15] Hwang ISS, Tang F. The distribution and gene expression of adrenomedullin in the rat brain: peptide/mRNA and precursor/active peptide relationships. Neurosci Lett 1999;267:85–8.
[16] Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H, et al. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. Biochem Biophys Res Commun 1993;192:553–60.
[17] Kitamura K, Sakata J, Kangawa K, Kojima M, Matsuo H, Eto T. Cloning and characterization of cDNA encoding a precursor for human adrenomedullin. Biochem Biophys Res Commun 1993;194:720–5.
[18] Kitamura K, Kangawa K, Kojima M, Ichiki Y, Matsuo H, Eto T. Complete amino acid sequence of porcine adrenomedullin and cloning of cDNA encoding its precursor. FEBS Lett 1994;338:6–10.
[19] Kono T, Sakai M. Molecular cloning and expression of preproadrenomedullin gene from common carp *Cyprinus carpio* L.. Gen Comp Endocrinol 2004;138:78–88.
[20] Lafont AG, Dufour S, Fouchereau-Peron M. Characterisation and distribution of calcitonin gene-related peptide in a primitive teleost, the eel *Anguilla anguilla* and comparison with calcitonin. Regul Pept 2004;117:141–8.
[21] López J, Cuesta N, Cuttitta F, Martinez A. Adrenomedullin in nonmammalian vertebrate pancreas: an immunocytochemical study. Gen Comp Endocrinol 1999;115:309–22.
[22] López J, Martínez A. Cell and molecular biology of the multifunctional peptide, adrenomedullin. Int Rev Cytol 2002;221:1–92.
[23] Lutz TA, Rossi R, Althaus J, Del Prete E, Scharrer E. Evidence for a physiological role of central calcitonin gene-related peptide (CGRP) receptors in the control of food intake in rats. Neurosci Lett 1997;230:159–62.
[24] Lutz TA. Amylinergic control of food intake. Physiol Behav 2006;89:465–71.
[25] Martínez V, Cuttitta F, Tache Y. Central action of adrenomedullin to inhibit gastric emptying in rats. Endocrinology 1997;138:3749–55.
[26] Muñoz M, Martínez A, Cuttita F, González A. Distribution of adrenomedullin-like immunoreactivity in the central

nervous system of the frog. J Chem Neuroanat 2001;21:105–23.
[27] Nag K, Kato A, Nakada T, Hoshijima K, Chandra Mistry A, Takei Y, et al. Molecular and functional characterization of adrenomedullin receptors in pufferfish. Am J Physiol Regul Integr Comp Physiol 2006;290:R467–78.
[28] Nag K, Kato A, Sultana N, Ogoshi M, Takei Y, Hirose S. Fish calcitonin receptor has novel features. Gen Comp Endocrinol 2007;154(1–3):48–58.
[29] Ogoshi M, Inoue K, Takei Y. Identification of a novel adrenomedullin gene family in teleost fish. Biochem Biophys Res Commun 2003;311:1072–7.
[30] Ogoshi M, Inoue K, Naruse K, Takei Y. Evolutionary history of the calcitonin gene-related peptide family in vertebrates revealed by comparative genomic analyses. Peptides 2006;27:3154–64.
[31] Parameswaran N, Spielman WS. RAMPs: the past, present and future. Trends Biochem Sci 2006;31:631–8.
[32] Poyner DR, Sexton PM, Marshall I, Smith DM, Quirion R, Born W, et al. The mammalian calcitonin gene-related peptides, adrenomedullin, amylin, and calcitonin receptors. Pharmacol Rev 2002;54:233–46.
[33] Reidelberger RD, Kelsey L, Heimann D. Effects of amylin-related peptides on food intake, meal patterns, and gastric emptying in rats. Am J Physiol Regul Integr Comp Physiol 2002;282:R1395–404.
[34] Roh J, Chang CL, Bhalla A, Klein C, Hsu SY. Intermedin is a calcitonin/calcitonin gene-related peptide family peptide acting through the calcitonin receptor-like receptor/receptor activity-modifying protein receptor complexes. J Biol Chem 2004;279:7264–74.
[35] Rossowski WJ, Jiang NY, Coy DH. Adrenomedullin amylin, calcitonin gene-related peptide and their fragments are potent inhibitors of gastric acid secretion in rats. Eur J Pharmacol 1997;336:51–63.
[36] Saita M, Shimokawa A, Kunitake T, Kato K, Hanamori T, Kitamura K, et al. Central actions of adrenomedullin on cardiovascular parameters and sympathetic outflow in conscious rats. Am J Physiol 1998;274:R979–84.
[37] Samson WK. Adrenomedullin and the control of fluid and electrolyte homeostasis. Annu Rev Physiol 1999;61:363–89.
[38] Sawada H, Yamaguchi H, Shimbara T, Toshinai K, Mondal MS, Date Y, et al. Central effects of calcitonin receptor-stimulating peptide-1 on energy homeostasis in rats. Endocrinology 2006;147:2043–50.
[39] Suzuki N, Suzuki T, Kurokawa T. Cloning of a calcitonin gene-related peptide from genomic DNA and its mRNA expression in flounder, *Paralichthys olivaceus*. Peptides 2001;22:1435–8.
[40] Takei Y, Hyodo S, Katafuchi T, Minamino N. Novel fish-derived adrenomedullin in mammals: structure and possible function. Peptides 2004;25:1643–56.
[41] Takei Y, Inoue K, Ogoshi M, Kawahara T, Bannai H, Miyano S. Identification of novel adrenomedullin in mammals: a potent cardiovascular and renal regulator. FEBS Lett 2004;556:53–8.
[42] Takei Y, Ogoshi M, Inoue K. A 'reverse' phylogenetic approach for identification of novel osmoregulatory and cardiovascular hormones in vertebrates. Front Neuroendocrinol 2007;28:143–60.
[43] Taylor GM, Meeran K, O'Shea D, Smith DM, Ghatei MA, Bloom SR. Adrenomedullin inhibits feeding in the rat by a mechanism involving calcitonin gene-related peptide receptors. Endocrinology 1996;137:3260–4.
[44] Taylor MM, Samson WK. A possible mechanism for the action of adrenomedullin in brain to stimulate stress hormone secretion. Endocrinology 2004;145:4890–6.
[45] Taylor MM, Bagley SL, Samson WK. Intermedin/adrenomedullin-2 acts within central nervous system to elevate blood pressure and inhibit food and water intake. Am J Physiol Regul Integr Comp Physiol 2005;288:R919–27.
[46] Taylor MM, Bagley SL, Samson WK. Intermedin/Adrenomedullin-2 inhibits growth hormone release from cultured, primary anterior pituitary cells. Endocrinology 2006;147(2):859–64.
[47] Thavanathan R, Volkoff H. Effects of amylin on feeding of goldfish: interactions with CCK. Reg Pept 2006;133:90–6.
[48] Van Rossum D, Hanisch UK, Quirion R. Neuroanatomical localization, pharmacological characterization and functions of CGRP, related peptides and their receptors. Neurosci Biobehav Rev 1997;21:649–78.
[49] Wei Y, Cao Y, Zhu Y, Chang J, Tang J. Immunohistochemistry and reverse transcription-polymerase chain reaction for detecting adrenomedullin in the central nervous system. Chin Med J 1998;111:793–6.
[50] Westermark GT, Falkmer S, Steiner DF, Chan SJ, Engstrom U, Westermark P. Islet amyloid polypeptide is expressed in the pancreatic islet parenchyma of the teleostean fish, *Myoxocephalus* (*cottus*) *scorpius*. Comp Biochem Physiol B 2002;133:119–25.
[51] Wimalawansa SJ. Amylin, calcitonin gene-related peptide, calcitonin, and adrenomedullin: a peptide superfamily. Crit Rev Neurobiol 1997;11:167–239.
[52] Wookey PJ, Lutz TA, Andrikopoulos S. Amylin in the periphery II: an updated mini-review. Sci World J 2006;6:1642–55.
[53] Young A. Role of amylin in nutrient intake—animal studies. Diabet Med 1997;14(Suppl. 2):S14–8.
[54] Young A. Tissue expression and secretion of amylin. Adv Pharmacol 2005;52:19–45.
[55] Zudaire E, Cuesta N, Martínez A, Cuttitta F. Characterization of adrenomedullin in birds. Gen Comp Endocrinol 2005;143:10–20.