Peptides 30 (2009) 803–807

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Short communication

Effect of calcitonin gene-related peptide (CGRP), adrenomedullin and adrenomedullin-2/intermedin on food intake in goldfish (*Carassius auratus*)

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ARTICLE INFO

Article history:
Received 13 November 2008
Received in revised form 18 December 2008
Accepted 18 December 2008
Available online 27 December 2008

ABSTRACT

The purpose of the present study was to elucidate the possible role of calcitonin gene-related peptide (CGRP), adrenomedullin (AM) and adrenomedullin-2/intermedin (IMD) on food intake regulation in goldfish (*Carassius auratus*). We examined the effects of intracerebroventricular (ICV) administration of these related hormones on food intake. Food-deprived goldfish were subjected to ICV injections of CGRP, AM and IMD and their food intake were quantified. CGRP at 10 ng/g body weight (bw) significantly decreased food intake as compared to saline-treated fish. IMD at 10 and 50 ng/g bw both significantly decreased food intake as compared to saline group. No significant differences were observed after AM administration. Our results suggest, for the first time in fish, a role for both CGRP and IMD in the central regulation of feeding in fish.

1. Introduction

In all vertebrates, the regulation of food intake is a complex process involving elaborate interactions between neurotransmitters, neuropeptides and hormones. The brain, in particular the hypothalamus, plays an important role as it contains networks that regulate energy homeostasis [7,48].

Calcitonin gene-related peptide (CGRP), adrenomedullin (AM) and adrenomedullin-2 (or intermedin, IMD), are members of the calcitonin/CGRP peptide family [27]. These peptides are structurally related [30] and exert their physiological functions through the calcitonin (CT) receptor-like receptor (CLR) complexed to specific receptor-activity-modifying proteins (RAMPs) [4,14,21]. CGRP-α (or CGRP) is a 37 amino acid neuropeptide derived from the tissue-specific alternative splicing of the calcitonin gene [1]. CGRP is widely distributed in the central and peripheral nervous systems and exerts a wide range of biological effects including neuromodulation, vasodilation and gastrointestinal regulation [34]. AM is a 52 amino acid peptide initially isolated from porcine adrenal medulla [11]. AM is produced in all tissues of the body, with the possible exception of the thyroid and thymus [10] and has a role in the regulation of nervous, endocrine, cardiovascular, renal and respiratory systems [17]. IMD/AM2 is a 47 amino acid peptide initially isolated in fish [28] and subsequently cloned by two independent groups in mammals [32,41]. IMD is expressed in brain, pituitary and most peripheral tissues [40], is involved in cardiovascular and body fluid regulations and has effects on the hypothalamic–pituitary–adrenal axis [9,41,44].

In mammals, CGRP, ADM and IMD have also been shown to be involved in the control of food intake. Intracerebroventricular (ICV) injection of CGRP decreases food intake [13,42] and gastric-acid secretion in rats [38]. Central administration of the CGRP receptor antagonist (CGRP (8–37)), elicits an increase in food intake suggesting that CGRP is a physiological feeding regulator acting on central CGRP receptors [18]. CGRP also may play a role as a peripheral satiety hormone because it is released from the gastrointestinal tract in response to a meal [51] or to increased glucose levels [8]. Peripherally administered CGRP decreases food consumption and suppresses gastric motility and acid secretion [23,31]. AM inhibits feeding and gastric emptying in rats when administered either peripherally or into the brain, both actions being mediated by CGRP receptors [20,31,33,43]. In rodents, both ICV and peripheral administration of IMD inhibit feeding, most likely through binding to CGRP receptors [45]. Peripherally administered IMD also suppresses stomach emptying responses in mice [32].

In fish, cDNAS encoding for CGRP, AM and IMD have been isolated in a number of species including flounder (*Paralichthys olivaceus*) [37], medaka (*Oryzias latipes*), pufferfish and zebrafish [27,28], rainbow trout (*Oncorhynchus mykiss*) [41], common carp

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0196-9781/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.peptides.2008.12.015

(*Cyprinus carpio*) [12] and goldfish [19]. The presence of each of these peptides has been detected in the brain, pituitary and most of peripheral tissues [6,12,15,16,19,28]. However, there is limited information on the biological functions of CGRP, AM and IMD in fish. It has been suggested that CGRP might have a role in osmoregulation [15] and in the regulation of gut motility [36] and AM might be involved in cardiovascular regulation [2]. We have recently reported the presence of CGRP, AM and IMD mRNA expression throughout goldfish brain, including in areas putatively implicated in the control of feeding, suggesting that these peptides may have a role in the control of food intake in fish [19].

In this study, we examined the roles of CGRP, AM and IMD in the regulation of food intake in goldfish by submitting fish to ICV injections followed by food intake quantification.

2. Materials and 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 (St John's, NL, Canada) under a simulated photoperiod of 16 h light/8 h dark in 60 l glass tanks, with constantly aerated and filtered water at 20 °C. The sides of the tanks were opaque to minimize external disturbances. Fish were fed a 1% wet body weight (bw) ration once a day (12:00), with commercially prepared 2.5 × 10 mm cylindrical trout pellets (Corey Aquafeeds, Fredericton, NB, Canada). Fish were acclimated under these standard conditions for a minimum of 2 weeks before the start of an experiment. 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. ICV injections

Brain ICV injections were administered following procedures described by Peter and Gill [29] and Volkoff et al. [49]. Briefly, following deep anesthesia in MS222 (Syndel Laboratories Ltd., Vancouver, British Columbia), a flap was cut in the roof of the skull using a drill equipped with a circular saw. The flap was then folded to the side, exposing the brain. 2 μl of saline or test solution was injected with a 5 μl Hamilton microsyringe into the brain third ventricle, using a specially designed stereotaxic apparatus, according to coordinates (+1.0, M, D 1.2) taken from the stereotaxic atlas of the goldfish brain [29]. Following surgery, the skull flap was secured by surgical thread. Fish were returned to their tanks and normally recovered from anesthesia within 2–5 min.

2.3. Observational experiments

For the evaluation of CGRP, AM and IMD effects on food intake, 5–15 animals were used for each treatment. For each experiment, two fish were placed in a single observation tank, to allow for an accurate observation of feeding behavior and food consumption, and were food deprived for 48 h. On the day of the experiment, an approximate 4% body weight ration of pellets per fish was administered 15 min post-injection. Behavioral observations and measurement of food intake commenced upon entry of pellets into the tank, and lasted 1 h. Experiments were carried out at the regular feeding time the fish had been adapted to (12:00). Food consumption was measured by counting the number of pellets eaten by each individual fish. Food consumption was converted to milligrams of food consumed/wet bw/time feeding based on the mean pellet weight fed to fish. Fish were tested in random order in terms of treatment and days. To verify that the injection procedures themselves did not influence feeding, food intake was assessed for unhandled fish as well as for control fish submitted to either anesthesia alone or sham operations, and compared to saline-treated animals.

2.4. Reagents

Human CGRP (Ala-Cys-Asp-Thr-Ala-Thr-Cys-Val-Thr-His-Arg-Leu-Ala-Gly-Leu-Leu-Ser-Arg-Ser-Gly-Gly-Val-Val-Lys-Asn-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Lys-Ala-Phe-NH2), human AM (Tyr-Arg-Gln-Ser-Met-Asn-Asn-Phe-Gln-Gly-Leu-Arg-Ser-Phe-Gly-Cys-Arg-Phe-Gly-Thr-Cys-Thr-Val-Gln-Lys-Leu-Ala-His-Gln-Ile-Tyr-Gln-Phe-Thr-Asp-Lys-Asp-Lys-Asp-Asn-Val-Ala-Pro-Arg-Ser-Lys-Ile-Ser-Pro-Gln-Gly-Tyr-NH_2) and pufferfish IMD 1 (Asn-His-Val-Met-Arg-Val-Ala-Cys-Val-Leu-Gly-Thr-Cys-Gln-Val-Gln-Asn-Leu-Ser-His-Arg-Leu-Tyr-Gln-Leu-Ile-Gly-Gln-Asn-Gly-Lys-Glu-Asp-Ser-Ser-Pro-Ile-Asn-Pro-His-Ser-Pro-His-Ser-Tyr-NH_2) were purchased from Phoenix Pharmaceuticals, Belmont, CA. Stock solutions of peptides were made in sterile water, aliquoted and stored at −20 °C. Aliquots were subsequently thawed and diluted in fish physiological saline [5] prior to use. Doses used were 5, 10 or 50 ng/g bw corresponding to 1.3, 2.6 or 13.2 pmol/g of CGRP, 0.8, 1.7 or 8.3 pmol/g of AM and 1, 2 or 10 pmol/g of IMD. These doses were chosen based on previous studies in mammals [43,45] and were similar to those previously used to test central effects of other peptides on feeding behavior in goldfish [46,47,49,50].

2.5. Statistics

Data were analyzed by one way analysis of variance (ANOVA) test followed by a Student–Newman–Keuls multiple range test for multigroup comparisons. A Student's *t*-test was performed in order to ascertain statistical differences between normalized mRNA expression in fed and fasting groups. A probability level of $p < 0.05$ was considered statistically significant. Data were analyzed using SPSS 14.0 software (SPSS Inc., IL, USA).

3. Results

Fig. 1 shows food intake after acute ICV injection of either saline or CGRP, AM and IMD at doses of 5, 10 and 50 ng/g bw. There were no significant differences in food intake between unhandled fish, fish submitted to either anesthesia alone or sham operations, and saline-treated animals (data not shown), confirming that the injection/surgery procedures themselves did not influence feeding. Only saline-treated fish were used as a control group in subsequent experiments. At 10 ng/g bw CGRP caused significant decrease in the number of pellets consumed during the 60-min observation period. Fish treated with CGRP at 5 or 50 ng/g bw had levels of feeding similar to that of saline-treated fish (Fig. 1A). There were no significant changes in food intake after ICV injection of 5, 10 or 50 ng/g bw AM compared to the saline group (Fig. 1B). Fish treated ICV with IMD at 5 ng/g bw had levels of feeding similar to that of saline-treated fish but both 10 and 50 ng/g bw doses significantly decreased food intake as compared to control group (Fig. 1C).

4. Discussion

In this study, we have shown that the injections of either human CGRP or pufferfish IMD 1 into the brain third ventricle of goldfish clearly induced a decrease in food intake. These results suggest, for the first time in fish, a role for CGRP and IMD in the regulation of feeding and corroborate in fish the anorectic action of CGRP and IMD described in mammals [31,32]. The reduction of feeding observed was not due to stress, since injected fish were responsive

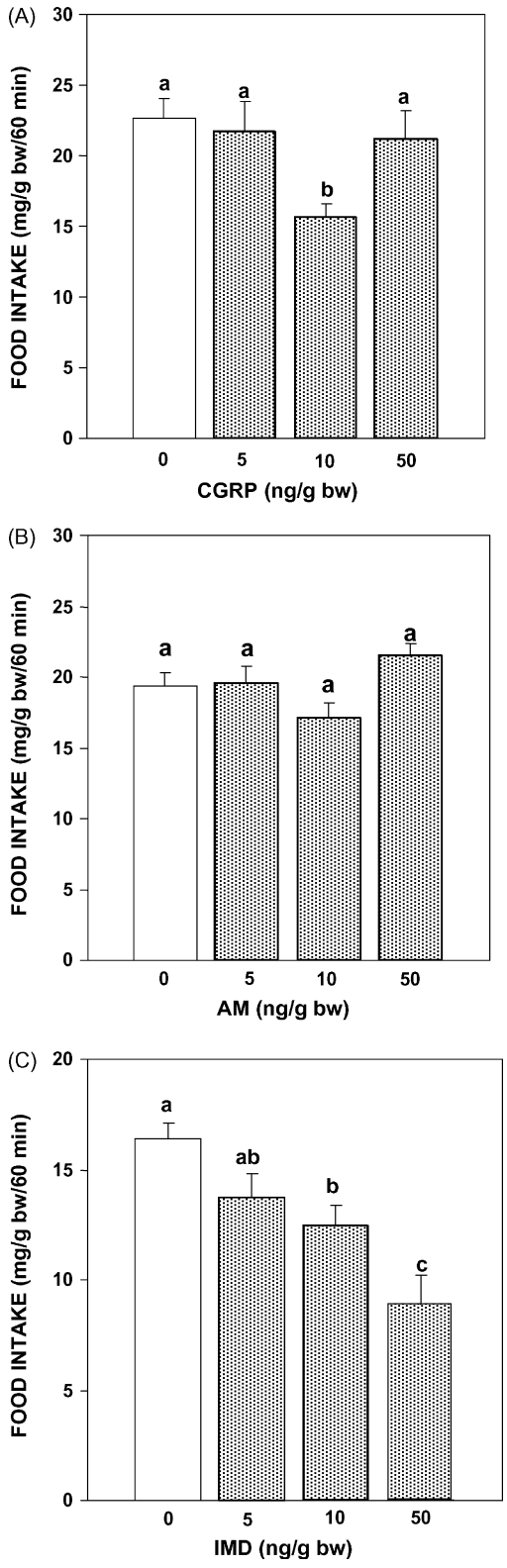

Fig. 1. Effects of ICV injection of human CGRP (A), human AM (B) and pufferfish IMD (C) on food intake of goldfish (*Carassius auratus*) 60 min following presentation of food. Fish were ICV injected with either saline (n = 9–13/group), CGRP at 5 (n = 6), 10 (n = 9), and 50 (n = 6) ng/g bw, AM at 5 (n = 8), 10 (n = 15), and 50 (n = 5) ng/g bw and IMD at 5 (n = 11), 10 (n = 12), and 50 (n = 8) ng/g bw. Fish received food 15 min post-ICV. Data are mean ± S.E.M. Bars with dissimilar superscripts indicate groups that differ significantly (CGRP; a and b are significantly different ($p < 0.05$); IMD; a, b and c are significantly different (a, b, $p < 0.05$; a, c, $p < 0.001$; ab, c, $p < 0.01$; b, c, 0.05). There are no statistical differences among groups that share common letters (ANOVA; $p < 0.05$).

to food, but ingested lesser amounts of pellets. Treated fish were also active and displayed a normal behavior.

To date, the role of CGRP as a central feeding regulator is well-established in mammals. CGRP and CGRP binding sites have been reported in brain areas related to feeding [34]. CGRP has been detected in the hypothalamus and CGRP neurons have been shown to project to the ventromedial hypothalamic nucleus, an area considered as one of the major brain satiety centers [26,35]. Lesions of the parabrachial area, which contains CGRP-expressing neurons, induce hyperphagia and obesity, whereas its stimulation causes hyperglycemia [25]. ICV injections of CGRP in rats decrease feeding and the central administration of a CGRP receptor antagonist (CGRP (8–37)) elicits an increase in food intake, indicating that CGRP is a physiological feeding regulator within the brain acting via central CGRP receptors [13,18,42]. The anorectic effects of CGRP observed following ICV injection in goldfish suggest the existence of CGRP and CGRP receptors in the brain. In fish, the presence of CGRP-like immunoreactive fibers in hypothalamus has been shown in teleosts species and elasmobranchs [3,22]. We have recently isolated cDNAs encoding for goldfish CGRP mature peptide and shown that CGRP mRNA is expressed in the whole brain and in discrete brain regions including the hypothalamus [19]. CGRP and its receptor have been cloned in flounder (*Paralichthys alivaceus*) and the expression of both genes has been detected in brain, suggesting that the neuropeptide may act in this tissue in a paracrine or autocrine manner [37].

In mammals, AM inhibits feeding and gastric emptying when administered either peripherally or into the brain, both actions being mediated by CGRP receptors [20,31,33,43]. In the present study, however, we found no changes in food intake in fish ICV injected with mammalian AM at any of the doses tested compared to the saline group. Therefore, the lack of significant effects of human AM on goldfish food intake exclude the possible central role on feeding in fish, the actions of this peptide being not conserved among vertebrates. It is possible, however, that a low affinity binding between the mammalian peptide and the goldfish receptor exist.

In the present study, IMD inhibited food intake when administered centrally in goldfish. To date, there are only two reports on the effects of IMD on food intake, both of them pertaining to rodents. In mice, peripheral administration of IMD inhibits food intake and suppresses stomach emptying responses [32]. A mammalian high-affinity receptor selective for IMD has not yet been identified as IMD is a non-selective ligand for complexes formed of CLR and RAMPs 1, 2 or 3 [32]. In mammals, both IMD-like immunoreactivity and mRNA expression have been reported in hypothalamus [39,45] and ICV administration of IMD decreases feeding, possibly through the activation of central CGRP receptors (CLR-RAMP1), suggesting that IMD acts within the central nervous system to inhibit food intake [45]. In fish, IMD has been characterized in several teleosts species [27,28,41] including goldfish [19]. In pufferfish and goldfish, IMD is expressed in the brain, pituitary and several peripheral tissues, including gut [19,28]. Similarly to CGRP, goldfish IMD mRNA is expressed in different brain regions, including the hypothalamus, supporting the present findings on the central role of IMD in the control of food intake [19]. Recently, a high-affinity IMD/AM2 receptor has been characterized in pufferfish (*Takifugu obscurus*) [24]. However, to date, little is known about the interactions of IMD with its receptor(s). Further studies on the expression analysis of IMD and its receptor will help understand the role of IMD in feeding regulation as well as in the modulation of other biological functions in fish.

When comparing the effects of CGRP and IMD on food intake in goldfish, both peptides significantly decrease food intake at doses of 10 ng/g bw (Fig. 1). The anorectic effects of CGRP were more

potent than that of IMD at 10 ng/g bw (31.22 and 24.20% food intake reduction with respect to the saline group after CGRP and IMD treatment, respectively). However, whereas the high dosage (50 ng/g bw) of IMD caused a pronounced decrease in food intake, fish treated with 50 ng/g bw of CGRP showed feeding levels similar to that of saline controls. Given the higher potency of CGRP, the food intake levels observed at the highest doses might be indicative of an overdose effect and a down-regulation of CGRP receptors. Similar results have been reported after ICV treatment with high doses of orexin in goldfish [47].

The effects of amylin, another calcitonin/CGRP peptide family member, on food intake have recently been reported in goldfish [46]. These experiments were carried following the same experimental protocol as the one used in the present work. Similar to CGRP and IMD, ICV injections of amylin at doses of 10 ng/g bw significantly decrease food intake to 50%, as compared to control fish. If we compare the percentages of feeding inhibition of amylin (close to 50%), CGRP (31.22%) and IMD (24.20%) in goldfish, the rank order of potency for the anorectic effects of these structurally related peptides (amylin > CGRP > IMD) is in line with potency comparisons previously established in rodents [31,32].

In summary, our results demonstrate that ICV administration of CGRP and IMD decrease food intake in goldfish, suggesting a role for both peptides in the central regulation of feeding in fish. These findings confirm the anorectic effects of these peptides as described in mammals and suggest a high conservation of CGRP and IMD actions throughout vertebrate phylogeny.

Acknowledgments

This work was supported by a postdoctoral fellowship Spanish Ministry of Education and Science to RM M-A, and by a Natural Science and Engineering Research Council of Canada (NSERC) Discovery grant to HV.

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