Isolation of the bioactive peptides CCHamide-1 and CCHamide-2 from Drosophila and their putative role in appetite regulation as ligands for G protein-coupled receptors

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INTRODUCTION

G protein-coupled receptors (GPCRs) constitute a large protein superfamily that shares a 7-transmembrane motif as a common structure. Human genome sequencing has identified several hundred orphan GPCRs for which ligands have not yet been identified (Vassilatis et al., 2003). GPCRs play crucial roles in cell-to-cell communication and are the most common target of pharmaceutical drugs. Therefore, the identification of endogenous ligands for orphan GPCRs will lead to clarification of novel physiological regulatory mechanisms and potentially facilitate the development of new GPCR-targeted therapeutics. Many bioactive molecules have been discovered or identified as endogenous ligands of orphan GPCRs through reverse pharmacology to date (Cocchi et al., 2012). These molecules include nocipeptin, prolactin-releasing peptide, orexin, apelin, ghrelin, metastin, and neuroedin S. The discovery of novel endogenous ligands for orphan GPCRs in mammals is currently challenging, possibly because of the restricted timing of expression or distribution of GPCR ligands. One orphan receptor in mammals is the bombesin receptor subtype 3 (BRS-3). BRS-3 plays a role in the onset of diabetes and obesity (Ohki-Hamazaki et al., 1997). Although several small molecules that are agonists and antagonists for BRS-3 have been synthesized, the native ligand of BRS-3 has not yet been identified (Majumdar and Weber, 2012). The recent sequencing of the Drosophila melanogaster genome has enabled the identification of at least 160 fly GPCRs (Studt and Crawshie, 2000). Drosophila is an excellent animal model for genetic analysis of developmental and behavioral processes, as it is a small, genetically modifiable organism with a relatively short lifecycle and can be bred easily under laboratory conditions. Structural or sequence comparison of newly discovered peptides in Drosophila with candidate molecules in mammals may lead to the discovery of new peptide signaling modules. We recently reported the discovery of diRYamide-1, diRYamide-2, and trissin as ligands

Abbreviations: BRS-3, bombesin receptor subtype 3; GPCR, G protein-coupled receptor.

Keywords: GPCR, novel bioactive peptide, Drosophila, CCHamide, bombesin receptor subtype 3

There are many orphan G protein-coupled receptors (GPCRs) for which ligands have not yet been identified. One such GPCR is the bombesin receptor subtype 3 (BRS-3). BRS-3 plays a role in the onset of diabetes and obesity. GPCRs in invertebrates are similar to those in vertebrates. Two Drosophila GPCRs (CG30106 and CG14693) belong to the BRS-3 phylogenetic subgroup. Here, we succeeded to biochemically purify the endogenous ligands of Drosophila CG30106 and CG14693 from whole Drosophila homogenates using functional assays with the reverse pharmacological technique, and identified their primary amino acid sequences. The purified ligands had been termed CCHamide-1 and CCHamide-2, although structurally identical to the peptides recently predicted from the genomic sequence searching. In addition, our biochemical characterization demonstrated two N-terminal extended forms of CCHamide-2. When administered to blowflies, CCHamide-2 increased their feeding motivation. Our results demonstrated these peptides actually present as the major components to activate these receptors in living Drosophila. Studies on the effects of CCHamides will facilitate the search for BRS-3 ligands.
An assay system using CG30106- or CG14593-expressing cells was maximum [Ca\textsuperscript{2+}] and then 50 μl of Calcium 4 assay kit reagent (Molecular Devices) for 1 h, subjected to determination of molecular weight by matrix-assisted laser desorption–ionization time of flight (MALDI-TOF) mass spectrometry by using a Voyager-DE PRO instrument (Applied Biosystems).

**MATERIALS AND METHODS**

**PURIFICATION OF Drosophila CCHamides-1 AND CCHamides-2**

An assay system using CG30106- or CG14593-expressing cells was prepared as previously described (Ida et al., 2011a,b). The full-length cDNA of CG30106 (GenBank accession number: NM_136355; residues 31 to 1700) and CG14593 (GenBank accession number: NM_136355; residues 656–2185) was obtained by RT-PCR using Drosophila cDNA as the template. The sense and antisense primers for CG14593 were 5′-aatagcgaggcagctacat-3′ and 5′-giggctgattcggtaactc-3′, respectively. The sense and antisense primers for CG14593 were 5′-ttggagttgcggcagggg-3′ and 5′-gggctgattcggtaactc-3′, respectively. The amplified cDNA was ligated into the pCDNA3 vector (Invitrogen). The expression vector, i.e., CG14593-pCDNA3-1, was transfected into Chinese hamster ovary (CHO) cells by using Fugene6 transfection reagent (Roche), and stably expressing cells were selected using 1 μg/ml G418. The selected cell line, i.e., CHO-CG30106-line 2-4 or CHO-CG14593-line 10-1, showed the highest expression of CG1306 or CG14593 mRNA, respectively. Cells were cultured in a humidified environment of 95% air and 5% CO\textsubscript{2}. Changes in intracellular Ca\textsuperscript{2+} concentrations ([Ca\textsuperscript{2+}]\textsubscript{i}) were measured using the FlexStation 3 fluorometric imaging plate reader to conduct high-throughput measurements of intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsuperscript{i}).

**CCHamides-1 AND CCHamides-2 cDNA as the template.** The sense and antisense primers for CG14593 were 5′-aatagcgaggcagctacat-3′ and 5′-giggctgattcggtaactc-3′, respectively. The amplified cDNA was ligated into the pCDNA3 vector (Invitrogen). The expression vector, i.e., CG14593-pCDNA3-1, was transfected into Chinese hamster ovary (CHO) cells by using Fugene6 transfection reagent (Roche), and stably expressing cells were selected using 1 μg/ml G418. The selected cell line, i.e., CHO-CG30106-line 2-4 or CHO-CG14593-line 10-1, showed the highest expression of CG1306 or CG14593 mRNA, respectively. Cells were cultured in a humidified environment of 95% air and 5% CO\textsubscript{2}. Changes in intracellular Ca\textsuperscript{2+} concentrations ([Ca\textsuperscript{2+}]\textsuperscript{i}) were measured using the FlexStation 3 fluorometric imaging plate reader to conduct high-throughput measurements of intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsuperscript{i}).

**CLONING OF Drosophila PREPRO-CCHamide-1 AND CCHamide-2 cDNA**

A BLASTn search of the Drosophila genome resources was performed by using sequence of the purified peptides, and we obtained D. melanogaster mRNA sequences [CG14358 (CCHA-

Reference:

Ida et al., 2011a, b.

**Ida et al.** (2012). *Isolation and function of CCHamides*. Neuroendocrine Science December 2012 | Volume 3 | Article 177 | 2
PROBOSCIS EXTENSION REFLEX TEST FOR APPETITE MEASUREMENT

The proboscs extension reflex (PER) test and feeding test were performed for the blowfly Phormia regina as previously described (Nusimura et al., 2005; Ida et al., 2011a). CG30106 was dissolved in blowfly food solution at a concentration of 10 μg/ml. Twenty flies were secured by their wings using washing pins, and the first PER test was performed by using 12 steps of sucrose concentrations that had been prepared to two-fold serial dilutions in distilled water, beginning from a sucrose concentration of 1 M. We investigated the PEr in three different groups of 20 flies each: no solution with or without peptide was injected into the shoulder of each fly. We repeated five sets of PER tests each, in which 20 flies were used in each batch.

STATISTICAL ANALYSIS

Results are presented as the mean ± SEM for each group. To compare the PER thresholds among the three groups, we used a non-parametric Steel–Dwass test. The criterion for statistical significance was p < 0.05 for all tests. The statistical software program GraphPad Prism (GraphPad software, CA, USA) was used for analyses.

RESULTS

STRUCTURAL DETERMINATION OF CCHamide-1 FOR CG30106

(Ca2+), assays were performed by using the gel filtration samples to isolate the endogenous ligands of CG30106 (Figure 1A). The active fractions were observed in eight sequential fractions (numbers 48–55). The fractions (51–55) with particularly high activity were separated by RP-HPLC. The active fraction of the P2 active fraction was purified as a single peak in the final RP-HPLC (Figure 2B-D). From the results obtained by using a protein sequencer and Drosophila cDNA encoding the purified peptides (Figure 2E), we deduced the primary structure of the peptide to be AQGSCAKKGCCQWVHGCHG-NH2 (P2), GCGQVHGCHG-NH2 (P3), and KGCGQVHGCHG-NH2 (P4, Figure 2G). All of these cysteines may form S–S bonds. The shortest peptide (P3) had been named CCHamide-2. The cDNA encoded a 136-residue protein (CG14375; Figure 2E) that contained features characteristic of an N-terminal signal peptide immediately preceding the purified longest peptide sequence (P2). All peptides were derived from the same precursor (CG14375), but the length of the N-terminal peptide was different. Mass spectrometric analysis revealed that the observed monoisotopic m/z values of the purified peptides (P2, 2216.80; P3, 1347.69; and P4, 1603.60) were similar to the theoretically predicted values (2216.99, 1347.52, and 1603.71, respectively) for a peptide that has an intrachain disulfide bond and C-terminal amidation. We generated the synthetic peptides AQGSCAKKGCCQWVHGCHG-NH2 (long-form CCHamide-2) and KGCGQVHGCHG-NH2 (CCHamide-2). The retention times of the P2 and P3 active fractions were identical to those of the synthetic AQGSCAKKGCCQWVHGCHG-NH2 and KGCGQVHGCHG-NH2 peptides (which have an intrachain disulfide bond) on RP-HPLC, respectively (Figure 2F). Thus, these data suggest that both natural peptides have an intrachain disulfide bond and C-terminal amidation.

PHARMACOLOGICAL CHARACTERIZATION

The interaction of CCHamide-1 and CCHamide-2 with CG30106 or CG14939 was examined using synthetic peptides. CCHamide-1 induced concentration-dependent, robust increases in [Ca2+]i in CHO-CG30106 cells, with a half-maximal response concentration (EC50) of 1.80 × 10−11 M (Figure 3A). CCHamide-2 potently activated CG30106 (EC50, 4.80 × 10−11 M (Figure 3A)). CCHamide-2 induced dose-dependent, robust increases in [Ca2+]i in CHO-CG14939 cells, with an EC50 of 4.80 × 10−11 M (Figure 3B).
FIGURE 1 | Purification of CCHamide-1 from fly extracts. Black bars indicate changes of \([Ca^{2+}]_i\) fluorescence signal in CHO-CG30106 cells. (A) G-50 gel filtration of the SP-III fraction of fly extracts. The active fraction was subjected to one step of CM-ion-exchange HPLC and three steps of RP-HPLC. (B) Final purification of the active fraction by RP-HPLC. (C) Nucleotide sequence and deduced amino acid sequence of CCHamide-1 cDNA. CCHamide-1 cDNA encode 182-residue peptides. The asterisk indicates a glycine residue that serves as an amide donor for C-terminal amidation. The CCHamide-1 sequence is underlined as (1). (D) Chromatographic comparison by RP-HPLC of natural CCHamide-1 and synthetic CCHamide-1. Black bar (P1) indicates the changes of \([Ca^{2+}]_i\) fluorescence signal in CHO-CG30106 cells. Each peptide was applied to a Symmetry C18 column (3.9 mm × 150 mm, Waters, MA, USA) with a 10–60% ACN/0.1% trifluoroacetic acid (TFA) linear gradient at a flow rate of 1 ml/min for 80 min. P1 represent active fraction containing natural CCHamide-1. (E) Active fractions of each chromatography and the amino acid sequence of CCHamide-1.

CCHamide-1 potently activated CG14593 (EC50: 3.32 × 10^{-8} M (Figure 3B)). Neither CCHamide-2 nor CCHamide-1 induced a response in CHO cells transfected with the vector alone (data not shown). In the investigation of the interaction between non-C-terminal amidated synthetic peptides or long-form CCHamide-2 and CG30106, the EC50 values were as follows: non-C-terminal amidated CCHamide-1, 1.66 × 10^{-10} M; non-C-terminal amidated long-form CCHamide-2, 8.93 × 10^{-9} M; long-form CCHamide-2, 6.45 × 10^{-9} M; and non-C-terminal amidated CCHamide-2, 1.22 × 10^{-7} M (Figure 3C). For CG14593, the EC50 values were as follows: long-form CCHamide-2, 1.49 × 10^{-10} M; non-C-terminal amidated long-form CCHamide-2, 1.18 × 10^{-8} M; non-C-terminal amidated CCHamide-2, 1.13 × 10^{-6} M; and non-C-terminal amidated CCHamide-1, 1.02 × 10^{-7} M (Figure 3D). PER TEST FOR MEASURING FEEDING SENSITIVITY As shown in Figure 4, a significant decrease was observed in the mean PER threshold, which was defined as the sucrose concentration at which 50% of flies show PER, after the injection of 10 pmol
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FIGURE 2 | Purification of CCHamide-2 from fly extracts. Black bars indicate changes of \([\text{Ca}^{2+}]_i\) fluorescent signal in CHO-CG14593 cells. (A) G-50 gel filtration of the SP-III fraction of fly extracts. The active fraction was subjected to one step of CM-ion-exchange HPLC and three steps of RP-HPLC. (B–D) Final purification of the active fraction by RP-HPLC. (E) Nucleotide sequence and deduced amino acid sequence of CCHamide-2 cDNA. CCHamide-2 cDNA encodes a 136-residue peptides. The asterisk indicates a glycine residue that serves as an amide donor for C-terminal amidation. The CCHamide-2 sequence is underlined as (4). The other long-form of CCHamide-2 is translated from (2) or (3). (F) Chromatographic comparison by RP-HPLC of natural CCHamide-2 and synthetic CCHamide-2. Black bars (P2, P3) indicate the changes of \([\text{Ca}^{2+}]_i\) fluorescent signal in CHO-CG14593 cells. Each peptide was applied to a Symmetry C18 column with a linear gradient elution for 80 min. P2 and P3 represent active fractions containing natural CCHamide-2. (b) Synthetic long-form of CCHamide-2. (c) Synthetic CCHamide-2. (D) Active fractions of each chromatography and the amino acid sequence of CCHamide-2.
FIGURE 3 | Pharmacological characterization of synthetic peptides using CG30106 or CG14593 stably expressed in CHO cells. (A, B) Concentration-response relationships of changes in [Ca^{2+}]_i for CCHamide-1 (open circle) and CCHamide-2 (open square) in CHO-CG30106 cells (A) or CHO-CG14593 cells (B). (C, D) Concentration-response relationships of changes in [Ca^{2+}]_i for various peptides, CCHamide-1 (open circle), and CCHamide-2 (open square) in CHO-CG30106 cells (C) or CHO-CG14593 cells (D). Non-C-terminal amidated CCHamide-1 (filled circle), non-C-terminal amidated CCHamide-2 (filled square), long form CCHamide-2 (open triangle), and non-C-terminal amidated long form CCHamide-2 (filled triangle). Each symbol on the line graph represents the mean ± SEM of data from six replicates for each experiment.
DISCUSSION

In this study, we biochemically purified 2 Drosophila peptides (CCHamide-1 and CCHamide-2) as endogenous ligands for Drosophila GPCRs CG30106 and CG14593. Recently, Hansen et al. (2011) independently identified these peptides from genome database and reported that synthetic CCHamide-1 and CCHamide-2 potently activated CHO/G16 cells expressing recombinant CG30106 and CG1493. Then, Reiher et al. (2011) characterized CCHamide-1 and CCHamide-2 from the Drosophila midgut by capillary offline RP-HPLC coupled with MALDI-TOF MS/MS. Our biochemical characterization, however, for the first time, demonstrated three forms of CCHamide-2. The CCHamide-2 preproprotein is 136 amino acid residues long and contains three forms of CCHamide-2. The CCHamide-1 preproprotein is 182 amino acid residues long and contains one form of CCHamide-1.

Pharmacological characterization by using CHO cells expressing GPCRs indicated that CCHamide-1 had a high potency for activating recombinant CG30106, but CCHamide-2 rather potently activated CG30106. In contrast, CCHamide-1 had a high potency for activating recombinant CG14593, but CCHamide-2 rather potently activated CG14593. Long-form CCHamide-2 and CCHamide-2 shared a highly similar potency for activating recombinant CG14593. Although we did not generate synthetic KKGCQA YGHVCYGGH-NH2, it is predicted to have a high potency similar to that of other forms of CCHamide-2 for activating GPCRs indicated that CCHamide-1 had a high potency

of CCHamide-2; the mean PER threshold decreased from 236 mM (30 min after linger solution injection) to 77.2 mM (30 min after CCHamide-2 injection, p < 0.05). In contrast, no difference was observed between the mean PER threshold without any injection and 30 min after linger solution injection (222 and 236 mM, respectively, p > 0.05).

FIGURE 4 | Effect of CCHamide-2 on PER of the blowfly. (A) Sigmoidal curves show the sucrose concentration-PER relationship for three fly groups: no injection (closed circle), injection with linger solution (open square), and injection of CCHamide-2 (open triangle). Each symbol on the line graph represents the mean ± SEM of data from five replicates for each experiment.

Therefore, in this study, we cannot conclude whether P4 and P2 be incomplete processing intermediates of GGQA YGHVCYGGH-NH2 (P3), originating from two alternative signal peptide cleavage sites and incomplete KR prohormone convertase processing. The quantity of the purified peptide could not be accurately measured at the time of the experiments. Because the gel filtration fractions with particularly high activity were separated by CM-ion-exchange HPLC at pH 6.3, we did not purify all peptides for their receptors from the flies collected. However, we purified peptide KKGCQA YGHVCYGGH-NH2 (P4) > AQGQA YGHVCYGGH-NH2 (P2) > GOQA YGHVCYGGH-NH2 (P3) in amount. Therefore, in this study, we cannot conclude whether P4 and P2 are mature peptides or incomplete processing intermediates of P3. Because both CCHamide-1 and CCHamide-2 have a disulfide bond and a YGH motif, the disulfide bond is predicted to be an important structure for GPCR activation. Additionally, both peptides have a GGX-NH2 motif at the C-terminus. Therefore, we synthesized non-C-terminus amidated peptides to determine whether the C-terminal amide was necessary for the activation of each receptor. These results show that these peptides are considered to require both disulfide bonds and C-terminal amides to activate their respective GPCRs. Because we biochemically purified these ligands for the receptors by using the reverse pharmacological technique, we propose that no further modified forms or unknown ligands exist for these receptors in the fruit fly. CCHamide-1 is a cognate ligand for CG30106 and the three forms of CCHamide-2 are cognate ligands for CG14593.

BRS-3 is a mammalian orphan receptor (Ohki-Hamazaki et al., 1997). Drosophila CG30106 and CG14593 belong to the BRS-3 phylogenetic subgroup (Heewo and Taghert, 2001). To provide new insights into the search for BRS-3 ligands, we examined whether CCHamides activate BRS-3, but we did not find any effect (data not shown).

CCHamide-1 and CCHamide-2 have been shown to be expressed predominantly in the brain and midgut (by FlyAtlas; http://www.flyatlas.org/; Chintapalli et al., 2007). In addition, CCHamide-1 and CCHamide-2 have been detected in the nervous system and midgut in a mass spectrometry study performed by Reiher et al. (2011). Therefore, CCHamides are suggested to be brain–gut peptides in insects. It is generally accepted that brain–gut peptides regulate feeding behavior in mammals (Williams et al., 2001). These peptides include neuropeptide Y, peptide YY, gastrin-releasing peptide, vasactive intestinal peptide, adrenomedullin, cholecystokinin, galanin, glucagon-like peptide-1, and neuromedin U (Zimanyi et al., 1998; Beck, 2001). In addition, CCHamide-2 was distributed in the larval fat body (by FlyAtlas). The insect fat body is a functional counterpart of the mammalian adipose tissue and liver (Gutierrez et al., 2007). In flies and certain other insects, the PER test has long been used to investigate behavioral sensitivity to phagostimulative tastes (Nismura et al., 2005). Flies extend their proboscis when the contact chemosensilla on their labella detects sweetness of sugar above a certain threshold concentration. Thus, we estimated the appetite or feeding motivation of the flies on the basis.
of the PER test for sucrose, in which the threshold concentration of sucrose was evaluated as an indicator of feeding sensitivity. The injection of CCHamide-2 decreased the threshold for feeding on a sucrose solution. These data suggest that CCHamide-2 stimulates the feeding motivation of flies. Indeed, administration of CCHamide-2 significantly increased the sucrose intake (Hiraguchi et al., 2010). In the presence of amino acids in the diet, target-of-rapamycin complex 1 (TORC1) signaling in fat cells generates a positive messenger that is released into the hemolymph (Colombani et al., 2003). This signal reaches the brain insulin-producing cells (IPCAs), where it remotely controls the secretion of Drosophila insulin-like peptides (Dilp). Insulin-like peptides couple growth, metabolism, longevity, and fertility with changes in nutritional availability (Grimmard et al., 2009). If CCHamide is a humoral factor that is secreted from the fat body like unpaired 2, it may play an important role in the modulation of nutrient status and growth (Rajan and Perrimon, 2012).

Mice lacking functional BRS-3 develop metabolic defects and obesity (Ohká-Hamazaki et al., 1997). Therefore, the natural ligand of BRS-3 is expected to be a prominent inhibitor of appetitive behavior. The difference between CCHamide and the unknown ligand for BRS-3 with regard to feeding behavior is not clear. Further studies should de-orphanize BRS-3 by considering CCHamide by using bioinformatics or antibodies for CCHamide or Drosophila GPCRs.

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