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25th October 2006

Professor Abba J. Kastin
Editor-in-Chief of Peptides
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Re Peptides-D-06-00265

Dear Professor Kastin A.J.

Thank you very much for your kind and qualified comments on our manuscript titled "Effects of ghrelin-related peptides on contractility of the isolated chicken gastrointestinal tract" (Peptides-D-06-00265). We have considered the comments and improved our manuscript as much as we could.

I am sending you our revised manuscript titled "Contractile effects of ghrelin-related peptides on the chicken gastrointestinal tract in vitro" by Kitazawa, Kaiya and Taneike. Title of the revised manuscript was changed following the comments of reviewer 2.

I would be grateful if the manuscript could be reviewed and considered for publication in Peptides.

Sincerely yours

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Contractile effects of ghrelin-related peptides on the chicken gastrointestinal tract in vitro

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Abstract

Ghrelin is an endogenous ligand for growth hormone secretagogue receptor (GHS-R), and it stimulates growth hormone (GH) release, food intake and gastrointestinal motility in mammals. Ghrelin has also been identified in the chicken, but this peptide inhibits food intake in the chicken. We examined the effects of ghrelin and related peptides on contractility of the isolated chicken gastrointestinal tract in vitro. Among ghrelin-related peptides examined (1 μM of rat ghrelin, human ghrelin, chicken ghrelin and growth hormone releasing peptide-6 (GHRP-6)), only chicken ghrelin was effective on contraction of the chicken gastrointestinal tract. Des-acyl chicken ghrelin was ineffective, suggesting that octanoylation at Ser^3 residue of chicken ghrelin was essential for inducing the contraction. Amplitude of chicken ghrelin-induced contraction was region-specific: highest in the crop and colon, moderate in the esophagus and proventriculus, and weak in the small intestine. The contractile response to chicken ghrelin in the crop was not affected by tetrodotoxin (TTX), but that in the proventriculus was decreased by TTX and atropine to the same extents. D-Lys^3-GHRP-6 (a GHS-R antagonist) caused a transient contraction and inhibited the effect of chicken ghrelin without affecting the high-K^+-induced contraction. Chicken ghrelin potentiated electrical field stimulation-induced cholinergic contraction without affecting the responsiveness to bath-applied carbachol in the proventriculus. The location of GHS-R differs in the crop (smooth muscle) and proventriculus (smooth muscle and enteric neurons). These results indicate that ghrelin has contractile activity on gastrointestinal tract in the chicken in vitro, and the effect was region-specific. The action would be mediated through the GHS-R, which is highly sensitive to chicken ghrelin.

Key words; Ghrelin, motilin, growth hormone secretagogue receptor, chicken gastrointestinal tract, contraction.

1. Introduction

Ghrelin, a 28-amino-acid peptide in which hydroxyl group on the side chain of the third residue, serine (Ser^3) is octanoylated, is an endogenous ligand for the growth hormone secretagogue-receptor (GHS-R) and was first isolated from the rat and human stomach. The octanoylation of Ser^3 is essential for the biological activity of ghrelin. Ghrelin is predominantly produced in the oxyntic mucosa of the stomach, with substantially smaller amounts derived from the intestine, pancreas, kidney, placenta, pituitary and hypothalamus [18]. Although ghrelin was initially discovered due to its growth hormone (GH)-releasing activity, this peptide is now considered to be an important regulator of glucose metabolism, insulin release, food intake, cardiovascular function and gastrointestinal function [19, 22]. The multifunctional roles of ghrelin are supported by evidence that GHS-R ligand binding sites and mRNA for GHS-R are widely expressed in the central nervous system and in several peripheral tissues [12, 21].

Ghrelin is structurally related to motilin and another group identified this peptide as motilin-related peptide [27]. Motilin is a gastrointestinal motility-stimulating peptide hormone synthesized at the mucosa of the small intestine (duodenum and jejunum) and is involved in the initiation of interdigestive migrating motor complex in the stomach [23]. Additionally, the structure of GHS-R has homologies with motilin receptor at several points, and these receptors constitute a new subfamily within class A of rodopsin like G-protein-coupled receptor [1, 22]. A lot of studies indicated the gastrointestinal contractility stimulating actions of ghrelin. In conscious rats and mice, ghrelin accelerates gastric emptying, enhances small intestine transit, and shortens the interdigestive migrating motor complex cycle [1, 6, 10, 17, 28]. In isolated muscle strips, ghrelin did not cause contraction of gastric strips directly but causes enhancement of the

nerve-mediated contraction [3, 6, 9, 17], and GHS-R was shown to be predominantly expressed in the enteric nerves but not in the smooth muscle cells [3, 30]. Therefore, ghrelin is thought to act as a neuromodulator of the enteric nervous system in mammalian gastrointestinal tract [22].

Ghrelin has also been isolated from non-mammalian species such as chicken, turtle and rainbow trout [13-15]. Chicken ghrelin is composed of 26 amino acids, has an octanoylated Ser^3, and has 54% total and 100% N-terminal sequence (Gly^1-Ser^2-Ser^3-Phe^4-Leu^5-Ser^6-Pro^7) identity with rat and human ghrelin. Chicken ghrelin and its mRNA are predominantly expressed in the proventriculus and are also detectable in the brain, lung and intestine [15, 29]. Chicken ghrelin also stimulates GH release in the chicken as seen in mammals [15]. However, in contrast to its action in mammals, ghrelin inhibits feeding, particular in neonatal chicks when injected intracerebroventriclulary [25]. This difference of action suggests that the physiological role of ghrelin is species-dependent and is different from that of mammals. Little is known about the actions of ghrelin in the chicken, other than its GH-releasing and anorexigenic actions. A recent molecular biological study demonstrated the expression of chicken GHS-R isoforms in central (pituitary, hypothalamus, telencephalon, cerebellum and brain stem) and peripheral tissues (ovary, kidney, proventriculus, duodenum and colon) [11]. The expression of ghrelin and its receptor in the chicken gastrointestinal tract and the well-known gastroprokinetic action of ghrelin in mammals prompted us to look at the effects of ghrelin on chicken gastrointestinal tract.

The aim of the present study was to study mechanisms of the contractile responses to ghrelin on different regions of the chicken gastrointestinal tract in vitro. To accomplish this, the effects of chicken, rat and human ghrelin and a synthetic peptidyl agonist for the GHS-R, growth hormone releasing peptide-6 (GHSR-6) on contractility of the isolated chicken gastrointestinal tract (esophagus, crop, proventriculus,

duodenum, jejunum, ileum and colon) were examined. Since chicken motilin has been shown to cause contraction of the proventriculus and ileum [16], region-related difference in contraction of motilin was also investigated and the effect was also compared with that of ghrelin. A neuromodulating role of ghrelin in nerve-smooth muscle transmission was also clarified by examining the effect of ghrelin on electrically evoked neural responses in the proventriculus.

2. Materials and methods

2.1. Animals and tissue preparations

All experiments were performed in accordance with institutional Guidelines for Animal Care at Rakuno Gakuen University (2006). Male white Leghorn chickens (*Gallus domesticus*), aged 2-4 weeks (80-120 g), were anaesthetized with ether, stunned, and bled to death. The esophagus, crop (located adjacent to the lower esophagus and storing foods), proventriculus (located next to the crop and corresponding to the gastric fundus in mammals), duodenum, jejunum, ileum and colon were removed after a midline incision, and their luminal contents were flushed out using ice-cold Krebs solution. The esophagus, crop, proventriculus and colon were cut open longitudinally, and smooth muscle strips in the longitudinal direction (1 mm in width and 10 mm in length) were prepared after cutting away the mucosa. In the case of the small intestine (duodenum, jejunum and ileum), longitudinal muscle strips attached myenteric plexues were prepared by peeling away longitudinal muscles using a cotton-wool swab and fine tweezers.

2.2. Contraction study

The smooth muscle preparations of the chicken gastrointestinal tract were suspended vertically in an organ bath (5 ml) to measure longitudinal muscle contraction. The organ bath contained warmed (37 ℃) Krebs solution (mM): NaCl, 118; KCl, 4.75; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $NaHCO_2$, 25 and glucose, 11.5 equilibrated with 95%O_2 + 5%CO_2 (pH, 7.4). Mechanical activity of the preparations was measured with an isometric force transducer (SB-11T, Nihon Kohden) and recorded on an ink-writing recorder. Initial loads were set at 0.5 g for each preparation. The preparations were rinsed with Krebs solution every 15 min and allowed to equilibrate for 1 h. Prior to the addition of peptide and electrical field stimulation (EFS), each strip was subjected to 3 or 4 stimulations with high-K^+ (50 mM) until a reproducible contraction was obtained. First, in order to examine whether or not ghrelin causes contraction of chicken gastrointestinal smooth muscles in vitro, ghrelin peptides (rat, human and chicken) and GHRP-6 at 1 μM were applied to muscle preparations obtained from different regions and the evoked responses were observed. In the crop and proventriculus preparations, a non-cumulative concentration-response curve was established with ascending concentration increments of ghrelin-related peptides at 45-min intervals. The amplitude of contractions among the preparations was normalized by a standard contraction of high-K^+ (50 mM) and expressed as a relative contraction. Effects of chicken motilin (1 μM), enough concentration to cause the maximum contraction [16], on smooth muscle preparations isolated from the chicken gastrointestinal tract were also examined. Region-dependent different amplitudes of contraction caused by ghrelin and motilin were compared.

To investigate modification of smooth muscle responsiveness to a muscarinic agonist by ghrelin, concentration-response curves to cumulatively applied carbachol in the proventriculus were constructed in the absence and presence of ghrelin-related

peptides (10-min treatment). $-\text{Log}EC_{50}$ (pEC_{50}) values, maximum contraction and slope factor of concentration-response curves were compared in the absence and presence of ghrelin-related peptides.

The effects of chicken ghrelin on EFS-induced contraction were examined using the proventriculus. Through two platinum electrodes placed on the left and right sides of the bath, sandwiching the preparations, EFS (5 Hz for 10 s, 0.5 ms in duration, and submaximum voltage of 15-20 V) was applied repetitively at 5-min intervals. After observing 2-3 reproducible EFS-induced contractions, ghrelin-related peptides (1 μM) were applied at the middle of stimulation interval and their effects on the contraction were observed.

2.3. Chemicals

The following chemicals were used in the present experiments: atropine sulphate (Wako, Osaka, Japan), carbamylcholine hydrochloride (carbachol, Sigma, St Louis, MO, USA), rat ghrelin (Peptide Institute Inc., Osaka, Japan), human ghrelin (Peptide Institute Inc., Osaka, Japan), growth hormone releasing peptide-6 (GHRP-6, Bachem, Bubendorf, Switzerland), D-Lys^3-GHRP-6 (Bachem, Bubendorf, Switzerland), 5-hydroxytryptamine hydrochloride sulfate complex (5-HT, Sigma, St Louis, MO, USA), ketanserin hydrochloride (Tocris, Ellsville, MO, USA), methysergide hydrochloride (Tocris, Ellsville, MO, USA) and tetrodotoxin (Wako, Osaka, Japan). Chicken ghrelin was custom-synthesized by Daiichi Asubio Pharma. Co. Ltd. (Gunma, Japan). Des-acyl chicken ghrelin was prepared from acylated chicken ghrelin: chicken ghrelin was incubated with 1N NaOH on ice for 1h, neutralized with the same volume of 1 M AcOH, and purified produced des-acyl ghrelin using reverse-phase high performance liquid chromatography. Chicken motilin was custom-synthesized by

Peptide Institute Inc. (Osaka, Japan).

2.4. Statistical analysis

The results of the experiments are expressed as means± S.E.M of more than four experiments. The significance of differences between the values was determined at $P< 0.05$ using Student's t-test (paired and unpaired) for single comparisons or ANOVA followed by Bonferroni Dunnett's t-test for multiple comparisons.

3. Results

3.1. Effects of ghrelin-related peptides and chicken motilin

Single application of 1 μM chicken ghrelin caused strong contractile responses in the crop and colon. Relative amplitudes of contractions were 71.5±5.2 % (crop, n=15) and 66.7±7.2% (colon, n=5) of 50 mM high-K^+-induced contraction. Other gastrointestinal muscle strips (esophagus, proventriculus, duodenum, jejunum and ileum) also responded to chicken ghrelin (1 μM), but the amplitudes of contraction differed depending on the regions (relative amplitudes of contraction: 37.9±8.7% (n=8) in the esophagus, 32.7±4.3% (n=9) in the proventriculus, 5.9±1.0% (n=5) in the duodenum, 22.8±3.2% (n=5) in the jejunum and 16.4±5.8% (n=6) in the ileum) (Figs. 1 and 2). On the other hand, the same concentration (1 μM) of rat ghrelin, human ghrelin and GHRP-6 was almost ineffective in inducing contractile response in the crop. Fig. 3 shows the concentration-response relationships for chicken ghrelin, rat ghrelin, human ghrelin and GHRP-6 in smooth muscle strips isolated from the

crop and proventriculus. Chicken ghrelin (10 nM – 1 μM) concentration-dependently caused contraction and the concentration of chicken ghrelin producing 50% of high-K^+-induced contraction was estimated to be 560±95 nM (n=6). Rat ghrelin, human ghrelin and GHRP-6 (10 nM-1 μM) did not cause contraction, but high concentrations of GHRP-6 (1-10 μM) contracted the crop preparation. The amplitude of contraction at 10 μM was 31.9±9.5% (n=4) (40% of 1 μM chicken ghrelin-induced contraction), suggesting a low sensitivity of this synthetic GHS peptide compared with chicken ghrelin. n-Octanoic acid modification at the third serine residue of mammalian ghrelin has been demonstrated to be essential for the biological activity of ghrelin in mammals [18]. Therefore, the effect of des-acyl chicken ghrelin on contractility of the crop and proventriculus was examined. Des-acyl chicken ghrelin (1 μM) was almost ineffective in causing the contractile responses, and the relative amplitudes of contraction were 4.2±0.7% (n=4) in the crop (chicken ghrelin: 71.5± 5.2%, n=15) and 2.1±1.1% (n=4) in the proventriculus (chicken ghrelin: 32.7±4.3%, n=9).

Motilin belongs to the same family of gut peptides with ghrelin [1, 22, 27] and has been shown to contract the proventriculus and ileal strips of the chicken trough activation of neural (proventriculus) and muscular motilin receptors (proventriculus and ileum) [16]. The effects of 1 μM chicken motilin on smooth muscle strips isolated from different regions of the chicken gastrointestinal tract were also examined to compare region-related different contractions of ghrelin and motilin. Chicken motilin (1 μM) also caused contraction of all of the gastrointestinal muscle strips examined. However, the amplitudes of contraction were different from region to region, similar to the case of chicken ghrelin. Relative amplitudes of contraction (chicken motilin, 1 μM) were 11±3.9% (n=5) in the esophagus, 29±2.8% (n=15) in the crop, 29±3.4% (n=14) in the proventriculus, 206±23% (n=5) in the duodenum,

139±15.4 % (n=5) in the jejunum, 106±5.8% (n=10) in the ileum and 58±20 % (n=4) in the colon (Fig. 2). The ranking order of motilin-induced contraction in the chicken gastrointestinal tract was quite different from that of ghrelin-induced contraction.

3.2. Pharmacological characterization of ghrelin-induced contraction in the crop and proventriculus

In order to clarify the contractile mechanisms, pharmacological properties of chicken ghrelin-induced contraction were examined both in the crop and proventriculus, because the effect of ghrelin was strongest in the crop (present study) and ghrelin has been shown to be predominantly localized in the mucosa of the proventriculus [15, 20, 29]. First, the contractile effect of chicken ghrelin was examined in the presence of tetrodotoxin (TTX, 1 μM), a blocker of the neural Na^+ channel. In the crop preparations, TTX did not significantly affect the amplitude of chicken ghrelin (1μM)-induced contraction (66.1±5.17%, n=7, *P*=0.62 vs. control value). In the proventriculus, on the other hand, TTX partially decreased the contractile response to chicken ghrelin (amplitude of contraction: 10.5±1.2%, n=5, *P*=0.004 vs. control value). Atropine (1 μM), a cholinergic muscarinic receptor antagonist, also significantly inhibited the chicken ghrelin-induced contraction (amplitude of contraction: 14.3±3.5%, n=4, *P*=0.029 vs. control value), and the remaining contraction was comparable with that in the presence of TTX (*P*=0.27, TTX vs. atropine).

Second, to clarify the involvement of GHS-R in the response to chicken ghrelin, the effect of pretreatment with D-Lys^3-GHRP-6 (a GHS-R antagonist) on the contraction induced by chicken ghrelin was examined. D-Lys^3-GHRP-6 (50 μM) applied to an organ bath caused a transient contraction of the crop preparations,

reaching a plateau within 1-2 min and recovering to the resting level within 10-12min. The amplitude of contraction (71.8±8.3%, n=8) was the same as that induced by 1 μM chicken ghrelin (71.5±5.2%, n=15). In the presence of D-Lys^3-GHRP-6 (for 15 min), chicken ghrelin (1 μM)-induced contraction was significantly decreased (28.3± 4.0%, n=6, *P*=0.00006), but high-K^+ (50 mM)-induced contraction (107±1.3%, n=6) was the same as the control (100%) (Fig. 4A). Since D-Lys^3-GHRP-6 is a related peptide of GHRP-6, a GHS-R agonist, it is possible that D-Lys^3-GHRP-6 acts as a GHS-R agonist and that the agonist-induced decrease in responsiveness can produce the inhibitory action of this peptide. Therefore, the effect of 1 μM chicken ghrelin, which induced the same contraction as that induced by 50 μM D-Lys^3-GHRP-6, on the chicken ghrelin-induced contraction was examined. Chicken ghrelin also caused a transient contraction and the tension reached the resting level within 15 min. In the presence of chicken ghrelin (1μM), successively applied chicken ghrelin (1 μM) caused a comparable contraction (64.2±15.3%, n=5, *P*=0.62) to the response in the absence of chicken ghrelin, suggesting that chicken ghrelin-induced decrease in responsiveness to chicken ghrelin did not occur in the crop. Recently, D-Lys^3-GHRP-6 has been reported to act on 5-HT_2 receptors to produce contraction in the rat stomach [7]. The possible involvement of 5-HT_2 receptor activation was also investigated in the muscle strips of the chicken crop. Ketanserin (1 μM) or methysergide (1 μM) significantly inhibited the contractile response to D-Lys^3-GHRP-6 (50 μM, relative amplitudes of contraction: 21±5.5% (n=10) in the presence of ketanserin and 36.6±5.7% (n=12) in the presence of methysergide) and 5-HT (3-10 nM) without affecting the high-K^+ (50 mM)-induced contraction. In spite of the presence of both D-Lys^3-GHRP-6 (50 μM) and ketanserin (1 μM), the relative amplitude of chicken ghrelin-induced contraction (29±6.9 %, n=4) was comparable to that in the presence of only D-Lys^3-GHRP-6 (28.3±4.0%, n=6). The inhibitory

effect of D-Lys^3-GHRP-6 was also investigated in the proventriculus. Pretreatment with the antagonist significantly attenuated the chicken-ghrelin induced contraction (2.0±0.5%, n=5) without affecting the contraction induced by high-K^+ (50 mM, 107±3.9%, n=4) (Fig. 4B). Different from the contractile response to chicken ghrelin in the crop, pretreatment with chicken ghrelin (1 μM for 15 min) itself significantly decreased the contractile response to chicken ghrelin (12.4±1.6%, n=4, *P*=0.017 compared with the values in the absence of chicken ghrelin), suggesting chicken ghrelin-induced decrease in the responsiveness to chicken ghrelin in the proventriculus.

3.3.Effects of ghrelin-related peptides on contractile response to carbachol

Modification of responsiveness to carbachol by ghrelin-related peptides was tested in the proventriculus. Carbachol caused a concentration-dependent contraction (1 nM-1 μM, pEC_{50}=7.29±0.02, maximum contraction=124.7±2.1% of 50 mM high-K^+-induced contraction, n=12), and the slope factor of the concentration-response curve was 1.44 ±0.15 (n=12). Atropine (300 nM) decreased but TTX (1 μM) did not change the contractile response to carbachol, indicating the involvement of musclular muscarinic receptors in the contractile response. In the presence of 1 μM rat ghrelin, chicken ghrelin or GHRP-6, concentration-response curves for carbachol were constructed and compared with the control. pEC_{50}, maximum amplitude of carbachol-induced contraction and slope factor of the concentration-response curve in the presence of each peptide were 7.26±0.05, 132±6.0% and 1.51±0.12 (n=5) (rat ghrelin), 7.31±0.05, 132±9.4% and 1.43±0.09 (n=5) (chicken ghrelin) and 7.24±0.05, 128±4.8% and 1.23±0.12 (n=5) (GHRP-6), respectively. These values were not significantly different from the control values.

3.4 Effects of ghrelin-related peptides on EFS-induced contraction

Since TTX and atropine partially decreased the contractile responses to chicken ghrelin in the proventriculus, chicken ghrelin was supposed to modify the EFS-induced neural contraction. EFS (5 Hz) applied for 10 s at 5 min intervals caused an atropine-sensitive reproducible contraction as previously reported [16]. Chicken ghrelin was applied to an organ bath at the middle of 5-min interval stimulation. EFS-induced contraction was normalized using the amplitude of EFS-induced contraction obtained 7.5 min before application of chicken ghrelin (Fig. 5). Chicken ghrelin (1 μM) caused a contraction and significantly potentiated the EFS-induced contraction but the effect was short-lasting. The relative amplitudes of EFS-induced contraction (5 Hz) in the absence (2.5 min before application) and presence of 1 μM chicken ghrelin (2.5 min after application) were 96±5.3% (n=5) and 121±8.7% (n=5, P=0.035 compared with the EFS-induced contraction in absence of chicken ghrelin), respectively. On the other hand, rat ghrelin and GHRP-6 at the same concentration (1 μM) were definitely ineffective in potentiating the EFS-induced contraction (Fig. 5).

4. Discussion

Ghrelin, an endogenous peptide for the GHS-R, stimulates GH release, food intake and gastrointestinal motility in mammals [19]. Ghrelin also stimulates GH release but inhibits food intake in the chicken [15, 25]. The different action of ghrelin in chicken on food intake prompted us to examine the effects of ghrelin on gastrointestinal motility of the chicken. In the present in vitro experiment, only chicken ghrelin caused contraction of isolated gastrointestinal smooth muscle strips in a region-dependent manner through

activation of neural and/or muscular GHS-R. The region-dependency of the response to chicken ghrelin clearly differed that of the response to motilin, belonging to same family of gut peptides.

In general, ghrelin was ineffective in increasing muscle tonus of mammalian isolated gastrointestinal strips (mouse stomach, mouse colon, rat stomach, rat distal colon and rabbit stomach) [3, 5, 6, 9, 17]. In chicken gastrointestinal smooth muscles, neither rat ghrelin nor human ghrelin (1 μM)was effective, but the same concentration of chicken ghrelin caused contraction (increase in muscle tonus). However, contractile response was not induced by des-acyl chicken ghrelin. These results suggest that GHS-Rs, which is highly sensitive to chicken ghrelin, are present in the chicken gastrointestinal tract and that octanoylation of Ser^3 is also essential for eliciting biological activity of ghrelin as reported in mammals [18,19]. In the previous in vivo experiments, GH-releasing activity of these ghrelin peptides (chicken, human and rat ghrelin) were almost equal in chicken and rat models, suggesting that the N-terminal 7 amino acids sequence (Gly^1-Ser^2-Ser^3-Phe^4-Leu^5-Ser^6-Pro^7), which retained the octanoyl group at Ser^3 and was identical among three ghrelin peptides, could act as the minimum active core of ghrelin action [15]. However, in the present in vitro study, only chicken ghrelin was active to cause contraction. Although amino acid sequences of rat ghrelin are 93% identical with those of human ghrelin, 12 amino acids of chicken ghrelin (position: 8, 9, 10, 11, 12, 15, 17, 18, 19, 22, 24, 26, 54% identity) differ from human ghrelin [15, 19]. Therefore, different contractile activity between chicken ghrelin and human ghrelin might be caused by the differences in middle and C-terminal structure of both peptides. Bowers has already suggested that the C-terminal part of the ghrelin molecule plays a role in completing the bioactive conformation of the intact ghrelin molecule [2]. In addition to the difference in amino acid sequences of ghrelin peptides, intra-organ (brain and gastrointestinal tract) heterogeneity for the GHS-R in

the chicken might also explain the different activity of ghrelin peptides between in vivo GH releasing study [15] and the in vitro contraction study.

Chicken ghrelin contracted the chicken gastrointestinal smooth muscles in a region dependent manner (crop > colon > esophagus > proventriculus > jejunum > ileum > duodenum). This suggests a region-related heterogeneous expression of GHS-R in the chicken gastrointestinal tract. Widespread expression of GHS-R mRNA in the chicken gastrointestinal tract has been reported, but detail distribution of GHS-R expression was not examined [11]. Since ghrelin was highly concentrated in the mucosa of the proventriculus [15,20,29], ghrelin released from the proventriculus might affect the motility of the crop and colon in both endocrine and paracrine fashion. A recent molecular biological study has indicated that the GHS-R is expressed equally in all parts of the human gastrointestinal tract [26]. Therefore, the expression pattern of GHS-R along the gastrointestinal tract might be species-specific. Motilin is a ghrelin-related gut peptide, and it has been known that the motilin receptor and GHS-R are highly homologous [1, 22]. Although mechanism of contraction was different between proventriculus (myogenic and neural responses) and ileum (myogenic response) [16], it is interesting to note that chicken motilin also caused contraction of chicken gastrointestinal tract in a region-dependent manner; highest in the duodenum, high in the jejunum, moderate in the ileum and colon, and weak in the esophagus, crop and proventriculus. The gastrointestinal regions where strong contractile response to chiken motilin was observed, are consistent with distribution of chicken motilin [4], suggesting autocrine/paracrine action of motilin in these regions. Taken together, the results suggest that region-specific differences exist in the mechanical actions of both ghrelin and motilin in the chicken gastrointestinal tract. Ghrelin mainly regulates contractions of the upper (crop) and lower (colon) gastrointestinal tract, while motilin principally regulates small intestinal motility. An in vivo functional study has demonstrated the physiological

roles of motilin as a mediator of rhythmic oscillating complexes in the chicken small intestine [24]. It is necessary to examine functional roles of ghrelin in chicken gastrointestinal motility in vivo.

Immunohistochemical studies have demonstrated that GHS-R is localized in the enteric nervous system but not in smooth muscle tissue of the guinea-pig, rat and human gastrointestinal tract [3, 30]. Consistent with the localization of GHS-R on enteric neurons, ghrelin modulates EFS-induced cholinergic contraction without affecting the smooth muscle tonus [3, 6, 9, 17] or causes contraction that is completely blocked by TTX or atropine [8]. In the chicken gastrointestinal tract, pharmacological properties of chicken ghrelin-induced contraction indicated that the localization of GHS-R was different in the crop and proventriculus. GHS-R was located in both smooth muscle tissues and enteric neurons of the proventriculus, but it was present only in smooth muscle tissues of the crop. The present results suggested region- and species-related different localization of GHS-R in the gastrointestinal wall.

D-Lys^3-GHRP-6 is known as the only GHS-R antagonist available at present. In the chicken crop and proventriculus, after D-Lys^3-GHRP-6 caused a transient contraction of muscle strips, but this peptide inhibited the chicken ghrelin-induced contraction without affecting the response to high-K^+. The inhibition of muscle contraction by D-Lys^3-GHRP-6, together with an ineffectiveness of des-acyl chicken ghrelin described earlier suggests that GHS-R mediates chicken ghrelin-induced contraction. Recently, it was reported that D-Lys^3-GHRP-6 cross-interacted with 5-HT_2 receptor, and induced pronounced contraction in the rat fundic strips [7]. In this study, the contractile response to D-Lys^3-GHRP-6 in the crop was partially inhibited by treatment of both ketanserin and methysergide, suggesting that 5-HT_2 receptors were also involved in the contraction of the chicken crop by D-Lys^3-GHRP-6. However, amplitude of chicken ghrelin-induced contraction in the presence of both D-Lys^3-GHRP-6 and ketanserin was

the same as that in the presence of D-Lys^3-GHRP-6 alone. This result indicates that blocking of 5-HT$_2$ receptor binding activity for D-Lys^3-GHRP-6 does not affect the inhibitory effect of this antagonist on chicken ghrelin. In contrast to the result obtained in the rat fundus [7], methysergide only partially inhibited the response to D-Lys^3-GHRP-6 in the crop, suggesting the involvement of other (non-5-HT$_2$ receptor) receptor mechanisms in the contractile response to D-Lys^3-GHRP-6. D-Lys^3-GHRP-6 was suggested to interact with other unknown receptors, development of more potent and specific GHS-R antagonist is necessary to characterize GHS-R pharmacologically.

In conclusion, chicken ghrelin caused contraction of chicken gastrointestinal tract in a region-dependent manner. It is highly possible that this activity is mediated through the GHS-R. The localization of GHS-R differs between the crop (only smooth muscle) and proventriculus (smooth muscle and enteric neurons). In the chicken, ghrelin preferentially regulates the motor activity of the upper and lower parts of the gastrointestinal tract through direct (myogenic) and indirect (neurogenic) action mechanisms.

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Figure Legends

Fig. 1

Each trace shows a representative contractile response to chicken ghrelin (1 μM, ●) in smooth muscle strips isolated from the esophagus, crop, proventriculus, duodenum, jejunum and colon of the chicken. Amplitude of contraction was normalized using a standard contraction induced by high-K^+ (50 mM, ○) and is shown in Fig. 2.

Fig. 2

Gastrointestinal region-dependent difference in amplitude of contraction induced by chiken ghrelin (open column) and chicken motilin (closed column). Effects of chicken ghrelin (1 μM) and chicken motilin (1 μM) on smooth muscle strips from the esophagus, crop, proventriculus, duodenum, jejunum, ileum and colon were investigated, and evoked contractions were expressed as a percentage to high-K^+ (50 mM)-induced contraction (Ordinate). Each column represents the mean of five or more experiments with SEM shown by a vertical line.

Fig. 3

Concentration-contraction relationships of ghrelin-related peptides in crop (A) and proventriculus (B) longitudinal muscle strips. Symbols show concentration-response curves for chicken ghrelin (●), rat ghrelin (■), human ghrelin (▲) and GHRP-6 (○) constructed by single application with 45-min intervals. Ordinate: amplitude of the contraction was expressed as a percentage to high-K^+ (50 mM)-induced contraction. Abscissa: concentration of peptides (logM). Each point represents the mean of five or more experiments with SEM shown by a vertical line.

Fig.4

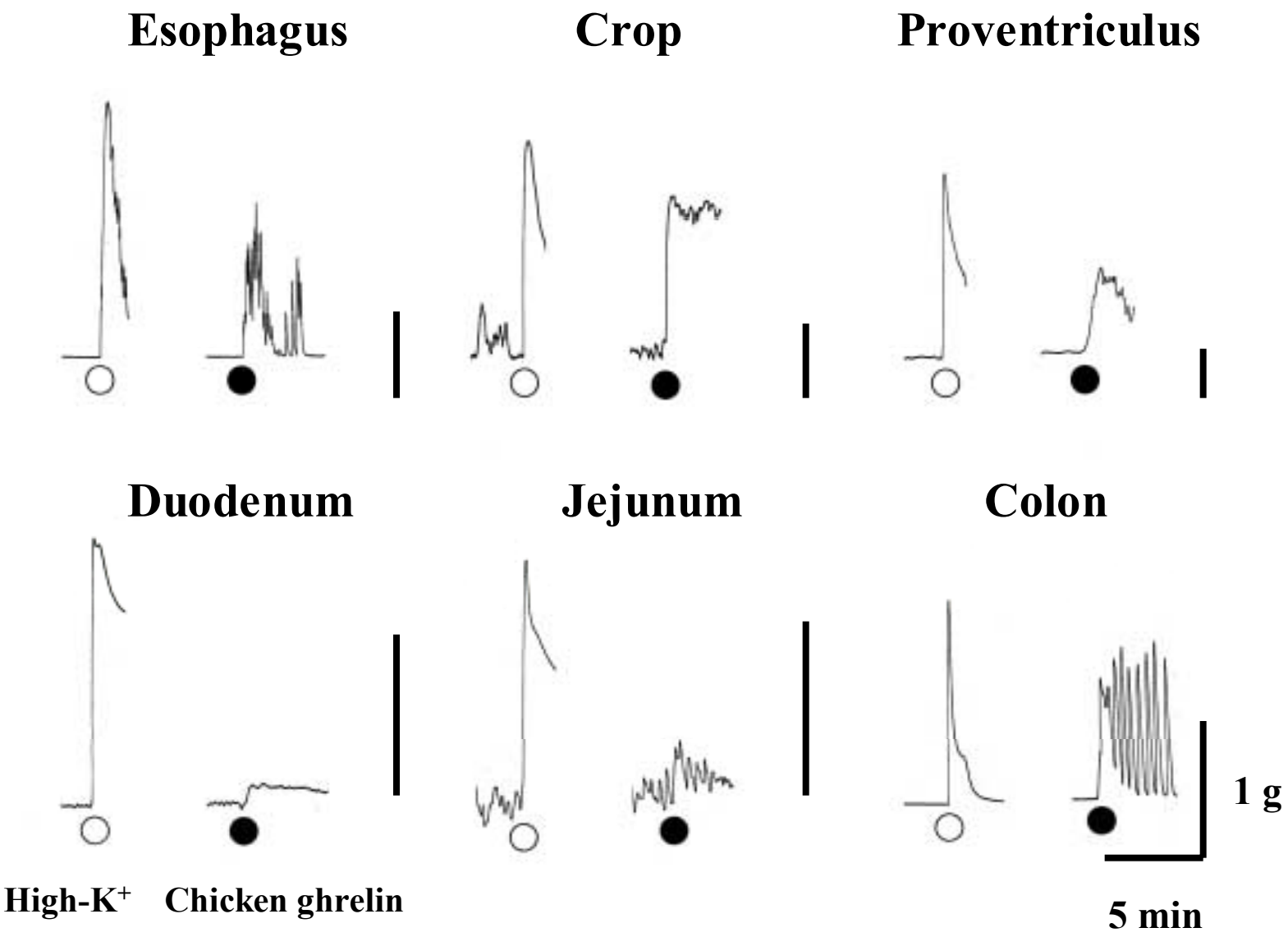
Effects of pretreatment with D-Lys^3-GHRP-6 on the contractile responses to chicken ghrelin and high-K^+ in smooth muscle strips of the crop (A) and proventriculus (B). After observing the control contractile responses to high-K^+ (50 mM, ○) and chicken ghrelin (1 μM, ●), crop and proventriculus muscle preparations were treated with D-Lys^3-GHRP-6 (50 μM, arrow) for 15 min. D-Lys^3-GHRP-6-induced contraction was transient, and the muscle tonus recovered to the resting level within 10-12 min. Chicken ghrelin and high-K^+ were applied in the presence of D-Lys^3-GHRP-6, and the contractile responses were compared with the responses in the absence of D-Lys^3-GHRP-6. In the lower traces (B), the vertical bar indicates 1 g for high-K^+-induced contraction and 0.5 g for chicken ghrelin and D-Lys^3-GHRP-6-induced contractions.

Fig.5

Potentiation of EFS-induced contraction by chicken ghrelin in muscle strips isolated from the proventriculus. A. Typical effect of pretreatment with chicken ghrelin (1 μM) on the contraction induced by EFS (5 Hz for 10 s at 5-min intervals, ▲). B. Effects of chicken ghrelin (1 μM, ●), rat ghrelin (1 μM, ▲) and GHRP-6 (1 μM, ○) treatment on the 5 Hz EFS-induced contraction of the proventriculus. Chicken ghrelin enhanced the EFS-induced contraction transiently, but rat ghrelin and GHRP-6 were ineffective. Peptides were applied at 7.5 min as indicated a black bar. EFS-induced contraction was normalized using the EFS-induced contraction 7.5 min before application of ghrelin peptides and expressed as relative contraction (ordinate). Abscissa: time (min) after the standard contraction of EFS in the absence of peptides. Each point represents the mean of five or more experiments with SEM shown by a vertical line. *; significantly different from EFS-induced contraction at 5 min.

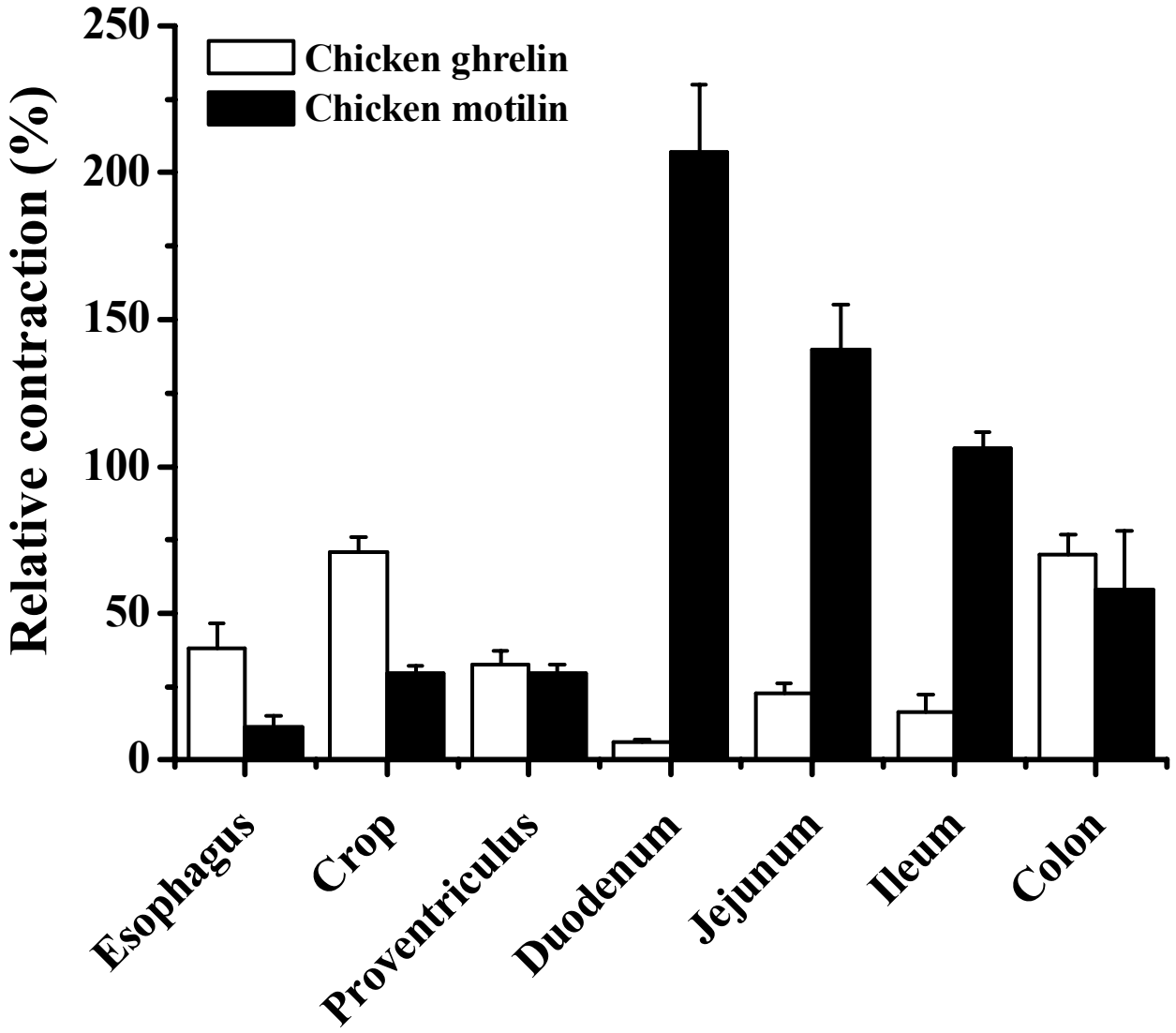
Figure

Fig.1 Kitazawa et al.



Figure

Fig.2 Kitazawa et al



Figure

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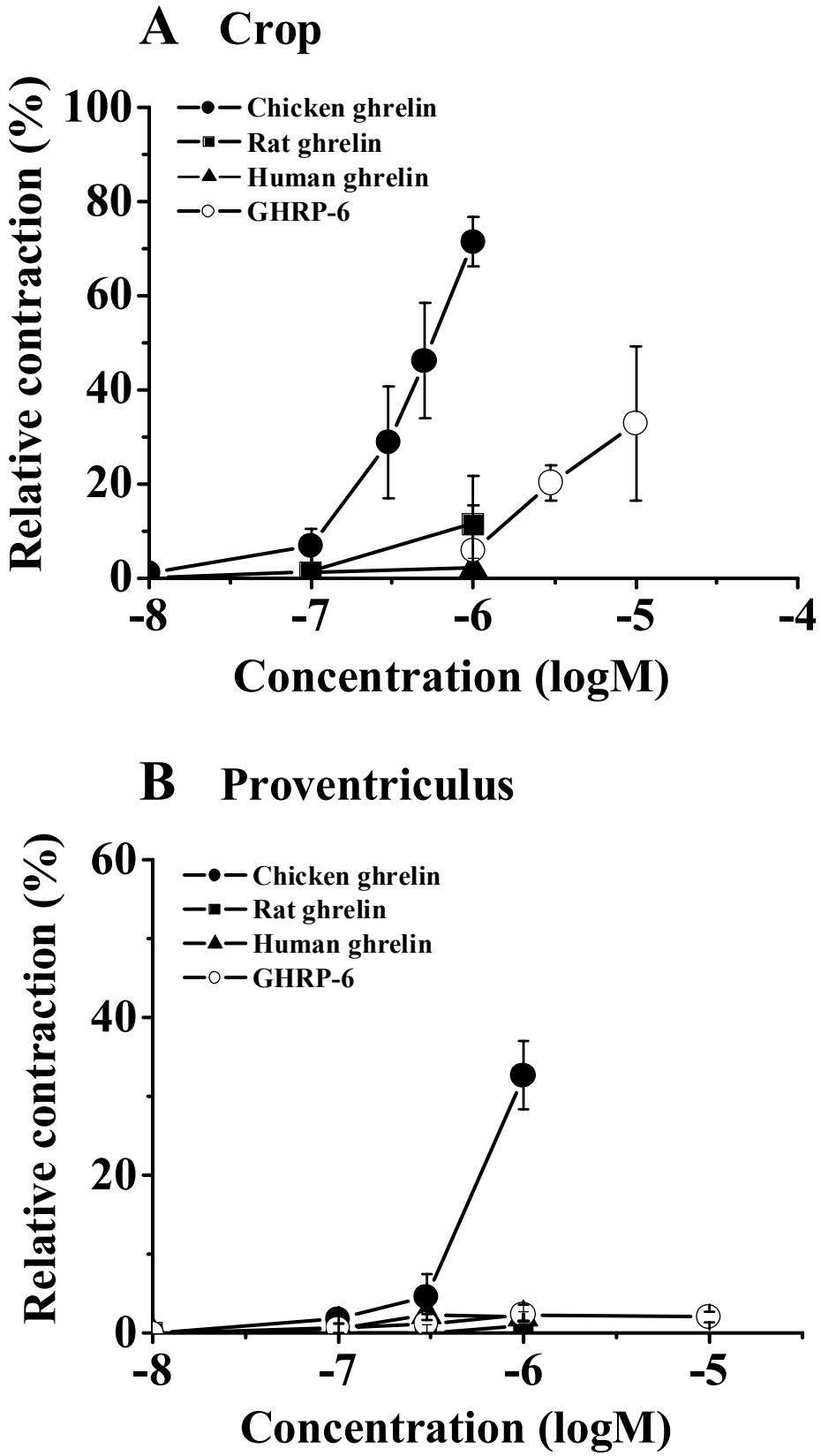

Figure

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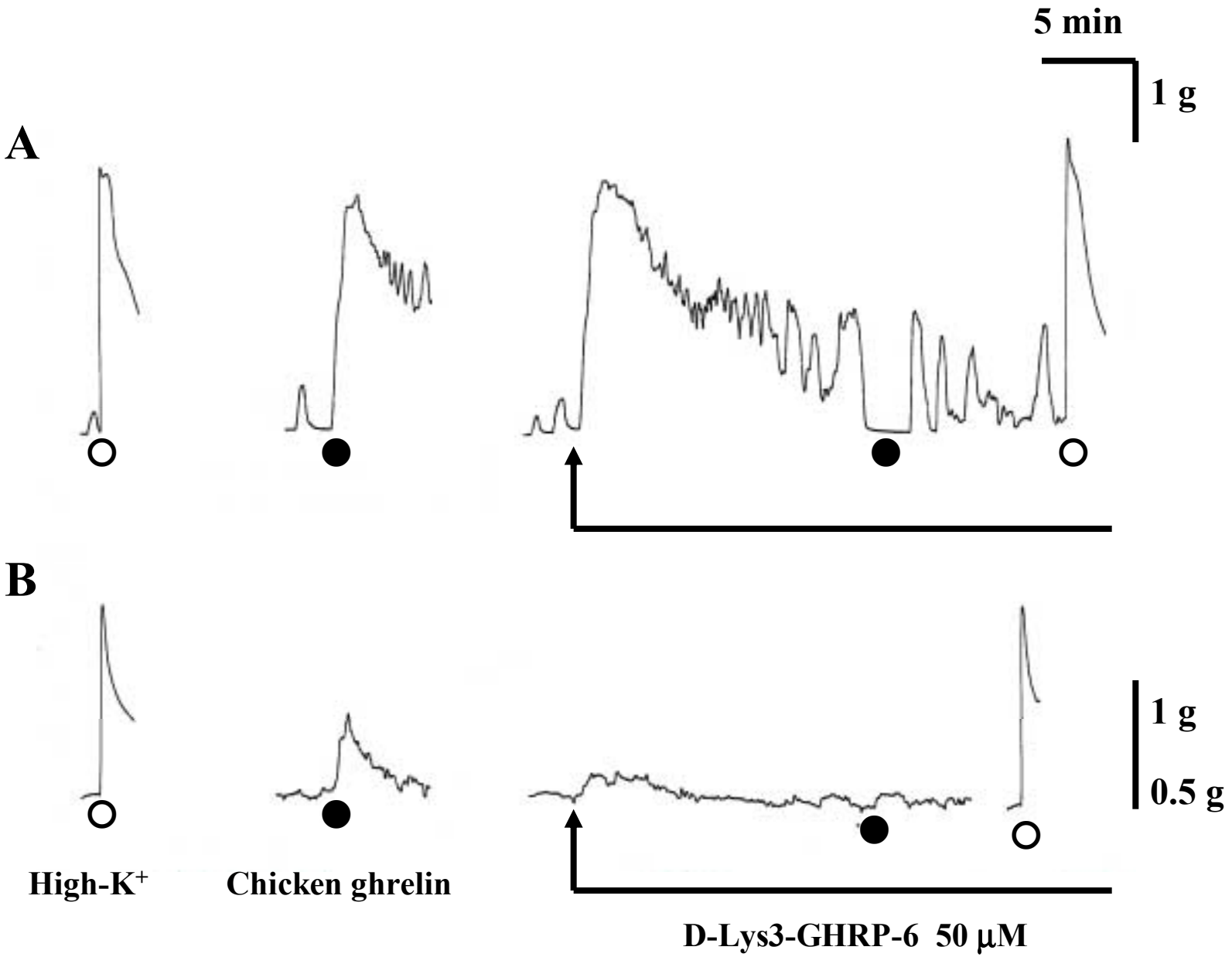

Figure

Fig. 5 Kitazawa et al.